Herbicide Effects On White Clover Growth and Nodulation.

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Abstract.

Five herbicides commonly used for suppression of weed growth in white clover seed crops were tested for toxicity against white clover (*Trifolium repens*), *Rhizobium trifolii* and the nitrogen fixing symbiosis formed between these two organisms. Trials were carried out on *R.trifolii* on solid and in liquid media to determine if growth of this bacterium was affected by the presence of the 5 herbicides. Paraquat and MCPB substantially inhibited bacterial growth on solid medium. Bentazone, fusilade and kerb caused very small zones of growth inhibition of *R.trifolii* on solid agar at high concentrations. None of the herbicides tested affected growth of *R.trifolii* in liquid culture.

In vitro studies of herbicide toxicity toward white clover were carried out to identify interactions of herbicide activity with rhizobial inoculation and supplied nitrogen, and to attempt to identify the targets of herbicide activity. Nodules grown under *in vitro* conditions were excised and used for ultrastructural examination.

Herbicides were applied to plants grown *in vitro* at two different stages of plant growth. White clover proved to be very sensitive to all herbicides at the early seedling stage. Three week old plants were more tolerant.

Pot experiments in a glasshouse environment indicated the relevance of *in vitro* experiments of herbicide toxicity against plants and gave information on the effect of soil water levels on herbicide activity.

Paraquat was extremely toxic to white clover both *in vitro* and in pot experiments. Nodulation is affected to some extent directly by this herbicide although dessication of foliage probably has some role in halting activity of the nitrogenase enzyme. MCPB caused severe deformation of root tissue both *in vitro* and in pot experiments. It must be either contaminated with the active form of this herbicide, MCPA, or is being broken down to the active form by bacterial or chemical action. Bentazone did not damage white clover or nodule activity in a consistent way *in vitro*. However this herbicide did have a deleterious effect on both plant weight and nodulation when applied to white clover grown in soil, particularly under conditions of low soil moisture. Fusilade showed a direct effect on the activity of nitrogenase *in vitro*. Fusilade also acted more severely against plants of higher nutritional status, and appeared to affect nodule activity directly rather than affecting nodules via damage to other plant parts. Kerb was very toxic to seedling white clover *in vitro* although older plants were not as susceptible and were stimulated by high concentration of kerb. In pots white clover was slightly inhibited by kerb at recommended concentration while 10 x this concentration did not cause any inhibition of nodulation or plant growth.

Differences in results between *in vitro* and pot studies of toxicity of these herbicides to white clover appear to be due to the different application methods used. *In vitro* herbicides were applied to the whole plant while in pot experiments herbicides were foliarly applied, hence more uptake by roots would be expected.

Pot experiments indicated that changes in nodulation generally reflected changes in plant growth rather than an independent activity of the herbicide on nodulation.

Chapter 1.0. Introduction - Leguminous Symbioses and Nitrogen Fixation.

1.1.Preamble.

To the best of present-day knowledge nitrogen fixation is confined to, though widespread amongst, prokaryotic organisms. It is found in both facultative and obligate anaerobes, autotrophs and heterotrophs, photosynthetic bacteria and blue-green algae (*Cyanobacteria*). Nitrogen fixers may be subdivided into two main groups, those that fix nitrogen in the free-living state and those that fix in symbiosis with plant or fungi. In terms of the total nitrogen fixed the symbiotic systems, particularly legumes are much more important to agriculture than are the free-living bacteria.

1.2.Rhizobia - Classification.

Microorganisms of the genus *Rhizobium* are aerobic, gram-negative soil organisms, classified on their ability to form nodules by infecting the roots of a member of the family Leguminoseae, or in the case of a non-infective (non-invasive) form, to have been definitely derived from a nodulating parent(Masterton and Sherwood 1970). Rhizobia are capable of an independent saprophytic existence in soil not containing legumes. They can survive in this condition for several years but become drastically reduced in number or will disappear in the continued absence of an appropriate host.

The genus *Rhizobium* is subdivided into six species and several other less well-defined groups based on host specificity. The four species *R.trifolii*, *R.leguminosarum*, *R.phaseoli* and *R.meliloti* are classified as fast growers having a mean generation time under optimal *in vitro* conditions of 3-4 hours. *R.japonicum* and *R.lupini* are classified as slow growers with a mean generation time of 6-8 hours under similar conditions. The further groups of rhizobia which do not fit easily into the 6 species, such as the "cowpea rhizobia" or "cowpea miscellany" and the "*Lotus* rhizobia" include both fast and slow growing organisms (Vincent 1977).

R.trifolii is specific for *Trifolium* species. The rhizobial bacteria multiply rapidly in the legume rhizosphere. The density of this population may have a direct effect on the number of infections (Masterton and Sherwood 1970).

1.3.Legumes - Classification and background.

With a few exceptions, symbioses involving rhizobial bacteria always have a member of the Leguminoseae as macrosymbiont. Legumes have a world-wide distribution and rank second or third amongst the flowering plants in the number of species they contain. Those species which are cultivated, include major food, forage and pasture plants, as well as some that produce timber and other products. The total global significance of legume-fixed nitrogen is obviously great, but impossible to define in strict quantitative terms.

There are two aspects of the benefits that can accrue from the use of nodulated legumes as crop or pasture plant. The first is the plants independance of soil nitrogen, the second is the potentially improved nitrogen status of the soil consequent on the use of the legume.

Legumes are generally intolerant of either very arid or waterlogged environments. It is thought that the nodules are ultrasensitive in this respect (Pate 1977). Long days promote development of more and larger nodules. A direct photosynthetic effect on symbiosis is believed to be responsible. It is clear that photosynthetic products fuel the energy demands of nodules and provide the acceptors by which newly fixed nitrogen is carried to the growing parts of the plant (Bergersen 1982). The principal short term effect of environmental stress is a reduction in nitrogen fixing ability of nodules. In the long term nodule shedding may be induced (Sprent 1976).

The inhibitory effect of high levels of combined nitrogen on nodulation is well known. It is thought root hair curling and subsequent formation of infection threads are more susceptible to injury from nitrogen treatment then are the later stages of nodulation. Nitrate and nitrite are more potent in this respect than ammoniacal forms of nitrogen. Nodulation often benefits from small supplements of fertilizer nitrogen applied at sowing or as the first nodules are forming (Richardson *et al.*1957).

SECTION A:

HERBICIDE TOXICITY

TO

WHITE CLOVER

GROWTH AND NODULATION

Chapter 2.0. Pesticide Effects on Rhizobia and Legumes - General Concepts.

2.1.Preamble.

At present an extensive range of pesticides are available for use against an even wider variety of agricultural pests. Domsch (1972) estimated that herbicides, insecticides and fungicides are used in the ratio of 4:2:1. The term "pesticide" will be used in this review to refer to any chemical used against insect, fungicide or plant pests in agriculture. Growing awareness of the possible side effects of pesticides has led to a large amount of research in this area. This, plus the influence of the rising costs of producing nitrogenous fertilizers, has focused attention on possible side effects toward the *Rhizobium*-legume symbioses. So many exacting processes are involved in achieving an effective symbiosis that even small amounts of toxic materials have the potential to render the association ineffective.

Pesticides may act on 4 processes of nodulation and nitrogen fixation;

(a)Survival of rhizobia.

(b)The infection process.

(c)Nodule formation and development.

(d)Nitrogen fixation in the nodule.

Pesticides may contact legumes and rhizobial bacteria in a range of agricultural situations.Pesticide residues may originate from soil treatment, seed dressings or foliar sprays applied to previous crops or to the legume crop itself. No one group of pesticides have been indisputably shown to affect Rhizobium-Legume symbioses more than another. Pesticide toxicity is dependant on chemical structure, environmental conditions and the sensitivity of the test organism. For effective analysis testing of toxicity must reflect the situation in which the pesticide is applied.

The primary considerations that influence decisions on how toxicity testing of pesticides on *Rhizobium*-legume symbioses should be carried out can be classified into 5 main groups.

(i)Effects on the microsymbiont vs. those on the macrosymbiont.

(ii)Variation due to differing pesticide formulations.

(iii)Variation due to differing pesticide concentrations.

(iv)*In vitro* vs. *in vivo* methods.

(v)Pesticide interactions with environmental stress.

2.2.Microsymbiont versus Macrosymbiont.

Generally pesticides affect the microsymbiont less than the macrosymbiont. Grossbard (1970b) exposed rhizobia to herbicides during growth, then used these cultures to inoculate white clover. The treated rhizobia were inoculated onto white clover plants both before and after being washed free of excess herbicide.

Unwashed inocula resulted in a decrease in nodulation and in some cases severe damage to the plants. Washed inocula did not exhibit any effect against plant growth or nodulation. Hence the pesticide activity was primarily against the plant and not the rhizobia. However some authors have identified the microsymbiont as the primary target of activity of DDT and organophosphorous insecticides (Brockwell and Robinson 1976;Appleman and Sears 1946).

2.3.Pesticide formulation.

Toxic activity may be displayed by the constituents of the formulation as opposed to the active ingredient itself. In the case of a commercial formulation of barban the solvent complex stimulated respiration and increased the number of microorganisms, perhaps through utilization of the compound as a food source. When pure and formulated aminotriazole were compared the formulated product exhibited greater adverse effect then did the pure compound on soil respiration and mineralization of nitrogen. When a commercial formulation was substituted by pure dinoseb the growth of *R.trifolii* tested on agar was slightly improved (Grossbard 1969/71). Lindstrom *et al.* (1985) found the toxic effect of dinoseb to *Trifolium pratense* was more pronounced when applied as a commercial formulation then as a pure compound.

Formulation and concentration of a pesticide varies between countries and localities. Comparisons of results for any particular pesticide is hindered by the use of local names for products without definition of actual active ingredients.

2.4.Pesticide concentration.

The concentration at which to apply a pesticide to rhizobia or a legume *in vitro* is very difficult to determine. The most desirable application rate is one equivalent to levels achieved by a particular pesticide in the field. In an *in vitro* system assumptions on spread of pesticides *in vivo* must be made. Penetration into soil must be allowed for in calculating application rates. Most researchers assume a penetration of approximately 5cm (Fisher and Hayes 1981;Greaves *et al.*1978). However it is the concentration in the soil solution that is most relevant to effects on microorganisms. This concentration will often be much lower due to adsorption to clay particles or organic matter and the limitation imposed by water solubility (Grossbard 1969/71).

The possibility that higher levels of pesticides can easily accumulate over several treatments, or are likely to concentrate in particular regions of the soil cannot be ignored. High levels of microorganisms are found in the rhizosphere which may be exposed to certain foliar pesticides following excretion from the roots (Grossbard 1969/71). Translocation within plants, for example, 2,4DB in *Lotus corniculatus* may concentrate pesticides in specific regions of the plant and thereby reach nodules at potentially dangerous concentrations (Garcia and Jordan 1969). Fletcher *et al.*(1956) highlighted the possibility that relative persistance of toxic substances in the soil may also have an important bearing on their toxicities.

A range of concentrations from below recommended field rates to well above are often applied. Vintikova (1965) stated "Herbicides were tested in considerably higher concentrations than are used currently in the treatment of [legume] stands. They nevertheless showed no abnormal inhibitory effect and it may therefore be assumed that they will exert no excessively harmful effect on the development of the rhizobial population in the soil." However interactions between a particular chemical and the soil environment may alter the chemical to a toxic form. The selectivity of the phenoxybutyric herbicides is based on the knowledge that not all plants have a *B*-oxidization system. Beta oxidation of the side chains of these chemicals converts them to an active form. Soil nocardias and other soil bacteria are known to beta oxidize these compounds to their active form and can thus render them toxic in soil (Webley *et al.*1958).

2.5.Comparisons between *in vitro* and *in vivo.*

The term *in vitro* refers to testing carried out on artificial media, *in vivo* is testing carried out in the natural environment. One of the greatest sources of divergent results obtained for toxicity of a particular pesticide is the use of pure culture experiments of toxicity of pesticides to microorganisms and plants, the results of which are extrapolated to soil conditions. Toxicity testing of pesticides in an *in vitro* situation was considered to give the pesticides "true" toxicity by Fletcher *et al.*(1956). These authors considered that high humidity in *in vitro* culture caused low transpiration and consequently little movement of the pesticides within the plant, resulting in lower toxicity of the pesticide.

Keckes (1970) found results obtained under laboratory conditions were opposite in most cases to the field experiments, as did Fisher *et al.* (1978). Diatloff (1970) found indications gained from *in vitro* methods were not always substantiated by field studies. However Borbely and Keckes (1972) found results of *in vitro* experiments on pesticide toxicity to rhizobia agreed completely with field results. Brockwell and Robinson (1976) concluded that results obtained from *in vitro* tests were indicative merely of the relative sensitivity of different organisms to various pesticides, and were not necessarily relevant to the reaction of the organism to the same compounds on seeds or on the plant. These studies highlight how misleading extrapolations from laboratory experiments to the field environment can be.

As stated by Fisher (1976) "Reduction of growth or metabolic activity of Rhizobium *in vitro* is not necessarily reflected in a decrease in symbiotic nitrogen so that it is hardly surprising that previous workers have found little correlation between laboratory and field experiments.".

Whilst pure cultures undoubtably have a vital role in providing information on the mode of action of pesticides on non-target organisms in particular, their use in determining whether the pesticide will have an effect in soil is rapidly losing favor. In the case of toxicity testing against a symbiotic association, *in vitro* studies do allow effects on the partners of the symbiosis to be assessed separately and hence are still of value in this respect.

2.6.Pesticide interactions with environmental stress.

Domsch (1978) suggested that the interaction of pesticides with natural stress situations would be a problem worthy of attention. Greaves and Malkomes (1980) also noted that humidity, temperature, pH and other soil factors were influences that may modify the response of organisms to pesticides. However Fisher *et al.*(1978) found soil pH was not a significant factor in any interactions of surfactant fungicides and white clover.

Research of pesticide effects on leguminous symbioses are of most value when crops are grown in nitrogen deficient environments particularly as legumes become increasingly used to replace nitrogen in over utilised soils. As *in vitro* experiments allow control of the environmental parameters, these methods will be used in the present study to investigate the possible interactions of pesticide application with different nitrogen sources.

Chapter 3.0. Pesticide Effects On Rhizobia - Concepts.

3.1. Preamble.

Fletcher and Alcorn (1958) stated "Generally [Rhizobial] bacteria are not affected by levels [of pesticides] applied to the soil". However the variety, frequency and quantities applied have increased dramatically since this time. Continual assessment of rhizobial tolerance to newly developed pesticides is essential to avoid unintentional damage of this bacterium.

Several reports of pesticide toxicity to rhizobia are contradictory. Kapusta and Rouwenhurst (1973) report that alachlor and trifluralin at 1 to 10 x recommended levels exhibited no effects on *Rhizobium japonicum*. However these herbicides at 0.5 to 5 times recommended rates inhibited *R.japonicum* growth in a study by Mallik and Tesfai (1983). From the literature it would appear there are 5 main factors to be taken into account in planning testing of pesticide toxicity to rhizobia. These are;

(i) Species/strain variation.

(ii) Resistant mutants.

(iii) Methods used.

(iv) Timing of treatments and measurements.

(v) Type of growth inhibition.

3.2.Species and strain variation.

Pesticide sensitivity varies depending on bacterial species and/or strain. Variation in sensitivity may be greater between strains of one species than between species themselves. Kaszubiak (1966) found fast growing species were more resistant to herbicides than slow growing ones. However Faizah *et al.*(1980) found sensitivity of rhizobia varied at the strain level. Fawaz *et al.*(1972) also found the sensitivity of 41 rhizobial strains to fungal seed protectants was a property of strain and to some extent species. Carbofuran affected growth of *R.leguminosarum* and *R.trifolii* but not *R.melilotii* and *R.japonicum* (Lin *et al.*1972). However *R.japonicum* was found to be more sensitive than *R.meliloti* or *R.trifolii* to 5 organophosphorous insecticides (Brockwell and Robinson 1976).

3.3.Resistant mutants of rhizobia.

The development of pesticide resistant mutants of rhizobia would allow otherwise toxic pesticides to be applied. This is especially important in the case of seed-dressings where likelihood of contact between rhizobial inoculum and the pesticide is very high. Kaszubiak (1968) found the development of resistance varied in different rhizobial strains. Rhizobia adapted to one herbicide showed acquired resistance to others. Adaptation of rhizobia to the herbicides used was not correlated with the ability to decompose these preparations.

Gillberg (1971) found repeated incubation in liquid media containing pesticide was the best way to obtain resistant mutants. The ability to infect leguminous plants was not affected in these pesticide resistant mutants. However Staphorst and Strijdom (1976) found that selection of rhizobium mutants resistant to toxic compounds frequently paralleled a decrease in the effectiveness of these mutants.

3.4.Methods of testing for side-effects of pesticides on rhizobia.

There are a variety of ways in which pesticide side-effects on rhizobia may be detected. Much of the testing is carried out *in vitro* on solid media. This provides a simple and easily quantified measure of toxicity.

Influence of pH and media composition on rhizobial sensitivity to herbicides was investigated by Kaszubiak (1966). Amino acids, vitamins, purines and pyrimidines present in the media and the pH of the media had no effect on rhizobial sensitivity. Grossbard (1970b) recorded that calcium presence or absence in the media did not lead to major differences in sensitivity of *R.trifolii* to ten commercial herbicides. Many authors have used filter paper discs on or wells in the agar to apply pesticide to plates seeded with bacteria. Inhibition zones give a measure of toxicity. A similar technique involves absorption of pesticides onto porcelain or glass beads which are then placed onto agar seeded with bacteria (Diatloff 1970). This method attempts to simulate the placement of pesticide dressings onto legume seeds.

Fisher (1976) incorporated pesticides into growth media and measured the rate of change in colony diameter as a parameter of toxicity. Kapusta and Rouwenhurst (1973) also incorporated pesticide into growth media as a wedge with untreated media poured on top to give a flat surface providing a concentration gradient of pesticide. Inhibition zones formed during rhizobial growth can be recorded visually. However Diatloff (1970) highlighted the fact that inhibition zones resulting from *in vitro* testing depend not only on the sensitivity of the test organism, but also on the concentration of the chemical and its ease of diffusion through the agar. Therefore these techniques may not give a true measure of toxicity.

Toxic effects of pesticides can be detected by growth measurements of rhizobia in liquid media via turbidometric means or viable plate counts. Shaking is required during growth to ensure aeration and even dispersal of the pesticide. Conditions provided for growth are optimal and are therefore very different from those in the soil. Variation in results have been recorded between *in vitro* methods. Differences in thiuram effect on *R.lupini* were observed in agar and liquid cultures (Jakubisiak and Golebiowska 1963). This variation was attributed to the different physical properties of the two media. However population dynamics are very different between cells in a colony and those in suspension, hence differences in growth is not suprising.

Mallik and Tesfai(1983) found the broth culture method of assay to be more sensitive then the paper disc method, although results of solid and liquid media

were comparable. Hence *in vitro* methods of measuring pesticide toxicity to rhizobia may only be used to indicate the existence of a possible toxic effect.

3.5.Timing of pesticide application and parameter measurements.

The timing of pesticide applications and growth measurements vary from 48 hours (Namdeo and Dube 1973) to 7 or more days (Diatloff 1986) depending on the growth characteristics of the particular strain. However Kaszubiak (1968) determined that the effect of several herbicides did not differ at different stages of growth of rhizobia.

3.6.Type of growth inhibition.

Inhibition of growth may be due to a bacteriostatic or a bactericidal effect. A bacteriostatic inhibition occurs when bacterial growth is halted but the bacteria remain viable. A bactericidal inhibition is when the bacteria can no longer divide. Bacteria have been observed to grow over inhibition zones (Tu 1980 ; Mallik and Tesfai 1983 ; Diatloff 1970). It is believed this indicates a bacteriostatic inhibition. Kaszubiak (1966) determined the smallest bactericidal dose of pesticides. Flasks of media were set up with increasing concentrations of pesticide. All were inoculated with rhizobia. After 24 hours incubation 0.2ml of the media from each flask was transferred to fresh untreated media and incubated for 7 days. Lack of bacterial growth in this fresh medium demonstrated that the action of the pesticide was bactericidal. However this technique has not been used widely in toxicity testing of pesticides against rhizobia.

Chapter 4.0. Literature Review of Pesticide Effects on Rhizobia.

4.1. Preamble.

A large part of the research on toxicity of pesticides to legume symbioses has concentrated on possible effects of pesticides on the microsymbiont. The effect of the pesticide may be to damage the microsymbiont and hence inhibit nodulation. As well as giving information on the dangers of using a particular pesticide this type of research may offer information on the mode of activity of a pesticide and on the way *Rhizobium* infects and establishes a symbiosis.

4.2. Herbicides.

(Refer Table 1.)

Vintikova *et al.*(1965) treated 7 species of rhizobial bacteria with 7 different herbicides on agar. The herbicides were ranked in order of toxicity, with MCPA as the most toxic and 2,4DB at a low concentration the least toxic. The respiration of *Rhizobium trifolii* was not inhibited by asulam, atrazine, endothal, lenacil, linuron, M&B 8882, paraquat, pyrazon or 2,3,6-TBA at 70ppm, however dinoseb did inhibit *R.trifolii* respiration at 70ppm (Grossbard 1970). Dinoseb was also recorded by Gillberg (1971) as inhibiting 3 species of rhizobia at 150ppm, whereas MCPA was resisted up to 800 ppm in liquid culture, a much higher level then that found toxic by Vintikova *et al.*(1965) on solid agar.

Triazines and halogen derivatives of aliphatic fatty acids were nontoxic to rhizobia, however phenoxy and pyridazine herbicides inhibited growth of some strains. Carbamate ureas and urea herbicides gave varying effects (Kaszubiak 1966). Responses of several species of rhizobium varied from mild stimulation by amitrole at 50 ppm to almost complete growth inhibition with other herbicides (paraquat, 2,4D-Na, 2,4D-amine, dalapon, 2,2DB and MCPB) (Namdeo and Dube 1973). However these levels are unlikely to be reached in the field. Manninger *et al.*(1972) treated 20 *Rhizobium* strains of 5 species with gramoxone (paraquat) and found the recommended field levels inhibited 40% of the strains *in vitro*.

Nineteen rhizobial strains were treated with a variety of pesticides on agar, including the herbicides dalapon, goal, roundup and tok E25. At recommended field levels the herbicide effects were minimal (Faizah *et al.*1980) however dalapon at 0.1mM was found by Sud *et al.*(1973) to stimulate growth of *Rhizobium leguminosarum* in broth culture, while 1mM had no effect and 10mM was found to inhibit growth.

Mallik and Tesfai (1983) treated 10 strains of *R.japonicum* with 3 herbicides *in vitro*. Metribuzin was non-inhibitory, alachlor and trifluralin inhibited rhizobial growth at 0.5, 1, 2 and 5 times the recommended rates. Trifluralin was recorded as more toxic than alachlor, in disagreement with the results of Kapusta and Rouwenhurst (1973) who found of 13 herbicides, 12 (chloramben, atrazine, alachlor, CDAA, DCPA, dicamba, linuron, naptalam, nitralin, propachlor, vernolate and trifluralin) were non-toxic at rates of 1 to 10 times the field concentrations to *R.japonicum in vitro*. Only chlorpropham (a carbamate herbicide) inhibited growth at

Table 1.
Herbicide Effects on Rhizobia.

The accepted common name as given in The Pesticide Manual of the British Crop Protection Council 6th ed.(1979) is used wherever possible.

If no common name is available the chemical name, trivial name or trade name is used.

Herbicide	Concentration	Media/Method	Species/Strain	Effect	Reference.
MCPB 2,4D aminotriazole TCA dinoseb chlorpropham dalapon	5 mg/disk 5 & 100 mg/disk. 3 mg/disk 50 mg/disk 10 mg/disk 3.5mg/disk 5 mg/disk	YMA Diffusion method	*R.leguminosarum*(6) *R.phaseoli*(4) *R.japonicum* *R.lupini* *R.trifolii* *R.meliloti* other Rhiz. sp.	Order of toxicity is MCPB>dinoseb(10 mg)>2,4-D(100mg)> 2,4D(5mg)>amitrole> chlorpropham>TCA> dinoseb>2,4DB(10mg)	Vintikova et al. (1965)
simazin prometryne antyperz dalapon pielik E 2,4 DB MCPB chloridazon dinoseb linuron monolinuron	10-10,000ppm diuron fluometron chloroxuron metobromuron lirobetarax chlorbufam	Growth in liquid culture by O.D.	*R.meliloti*(7) *R.trifolii*(1) *R.leguminosarum*(2) *R.japonicum*(1) *R.lupini*(2)	Resistance varied at strain level.	Kaszubiak 1966
asulam atrazine dinoseb endothal lenacil linuron M&B 8882 paraquat chloridazon2,3,6-TBA	70ppm	Growth in liquid media.	*R.trifolii*	Respiration was not inhibited by any except dinoseb which arrested oxygen uptake of cells.	Grossbard 1970
dinoseb MCPA	0-150ppm 0-1000ppm	G:1 medium growth by O.D.	*R.meliloti*(2) *R.legumino-sarum*(2)	All strains were inhibited by dinoseb.MCPA inhibited at > 400ppm.	Gillberg 1971.
neburon &monolinuron prometryne prometryne+simazine	Not given	YMA. Diffusion method	*R.lupini* *R.japonicum* *R.meliloti* *R.trifolii* *R.phaseoli* *R.legumino-sarum*	Order of toxicity is monolinuron> prometryne> prometryne+simazine> neburon	Borbely & Keckes 1972.
paraquat	750-800000ppm	Czapeks media. Diffusion method.	*R.japonicum*(2) *R.meliloti*(5) *R.trifolii*(7) *R.legumino-sarum*(6)	Field conc.'s inhibited growth of 40% of strains resistance varied at strain level.	Manninger et al. 1972

Herbicide	Concentration	Media/Method	Species/Strain	Effect	Reference.
dalapon paraquat	0-2000ppm	YEM	Rhizobia isolated from 3 soils.	Paraquat doubled lag phase & lengthened the log phase. Dalapon reduced stationary phase of fast growers.	Namdeo & Dube. 1973
chloramben atrazine alachlor chlorpropham CDAA DCPA dicamba linuron naptalam nitralin propachlor trifluralin vernolate	15ppm 15ppm 7.5ppm 15ppm 20ppm 52.5ppm 6ppm 7.5ppm 26ppm 7.5ppm 20ppm 5ppm 15ppm	YMA. Gradient plate technique.	*R.japonicum*	Chlorpropham inhibited growth. Others had no effect.	Kapusta & Rouwenhurst 1973.
TCA dalapon	0.1,1 & 10mM	Asparagine-mannitol broth.O.D.	*R.legumino-sarum*	0.1mM stimulated growth.1mM had no effect.10mM inhibited growth.	Sud et al. 1973.
hydrouracil-5,5-dichloro-3-isopropyl-6-methoxy-6-(trifluoromethyl)	10-400ppm	Rhizobium Y broth.(O.D) Diffusion methods.	*R.japonicum*(2)	Growth stimulated at high conc.'s near disc's.	Nelson & Hendrick 1976.
dalapon goal glyphosate nitrofen	72600ppm 200ppm 12100ppm 200ppm	YMA Diffusion methods.	Isolates from 19 leguminous hosts.	At field levels effects were minimal.	Faizah et al.1980.
alachlor metribuzin trifluralin glyphosate 2,4D	0.5-5 X recommended rates(5 to 250 micrograms/disk.)	YMA diffusion method.Liquid broth,growth by O.D.	*R.japonicum*(10)	Order of toxicity: trifluralin>alachlor> metribuzin. 2,4-DB at 25 micrograms reduced growth.	Mallik & Tesfai 1983.

these levels. An experimental herbicide was found to stimulate growth of *R.japonicum* on solid and in liquid culture (Nelson and Hendrick 1976), these authors suggested the bacteria could utilize the herbicide as a carbon source.

4.3. Fungicides.

(Refer table 2.)

Most seed and soil applied fungicides are toxic to *Rhizobium* in culture and therefore have the potential to limit *Rhizobium* survival on seed and hence nodulation establishment or activity. Keckes(1970) tested 6 species of rhizobia for susceptibility to a variety of fungicides. The fungicides were ranked in order of toxicity phygon > captan > thiram > spergon > ceresan > panogen. Diatloff (1970) applied fungicides on hollow porcelain beads to agar plates seeded with *R.japonicum*. The fungicide toxicities were ranked as ceresan > thiram > captan > chloranil which is the reverse order of captan and ceresan found by Keckes (1970). Both experiments were very similar, however concentrations of fungicides applied were not given by Keckes (1970), hence comparisons cannot be made to determine if this was the cause of this variation.

Fawaz *et al.*(1972) ranked the toxicity of fungicidal seed protectants to 41 rhizobial strains as ceresan wet, thiram and ceredan-T > thizoctol and ceresan > captan and dithane M45 > spergon > antracol > karathane. This order of toxicity disagrees with those of Keckes (1970) and Diatloff (1970). Fisher and Hayes (1981) found fenarimol and oxycarboxin affected growth of *R.trifolii* at low concentrations. Captan, dodine and oxycarboxin all caused marked reductions in growth of *R.trifolii*. Ethylen CP caused some growth inhibition. Benomyl, carboxin, dimethirimol, ethirimol, thiram, tridemorph, triforme and thiophanate methyl had no effect at concentrations ranging from 10 to 1000ppm (Fisher 1976).

Both strains of *R.trifolii* tested by Gillberg (1971) were inhibited by 50 ppm captan and 200 ppm neovoronit. *R.japonicum* was not affected by dyrene (a S-triazine fungicide) (Kapusta and Rouwenhurst 1973) but was damaged by other fungicides in the order thiram > captan > zineb > chlorothalonil, dodine, folpet and benomyl > carbendazin, pyroxychlor and quintozene (Tu 1980). Tu (1977) also recorded thiram as being extremely toxic to *R.japonicum* at 0.1 to 10 times the recommended levels.

Mallik and Tesfai (1983) ranked the toxicity of the fungicides they tested as carboxin > mancozeb > thiram > captofol > captan > fenaminsulf and PCNB which were non-toxic, which is in agreement with the order of toxicity of captan and thiram found by Tu (1980). Fungicides recommended for seed dressings were evaluated for toxicity to 2 cowpea rhizobia. The order of toxicity found was vitavax (=carboxin), thiram, dithane SPC, manzate and TCMTD >> captan, botran, defolaton and saadtan >> terracoat E205, brassicol, benlate, dexon and HPMTS (Staphorst and Strijdom 1976). These authors ranked thiram and captan in the opposite order of toxicity as Tu (1980) and Mallik and Tesfai (1983).

Table 2.
Fungicide Effects on Rhizobia.

The accepted common name as given in The Pesticide Manual of the British Crop Protection Council 6th ed.(1979) is used wherever possible.
If no common name is available the chemical name, trivial name or trade name is used.

Fungicide	Concentration	Media/Method	Species/Strain	Effect	Reference
thiram chinonoxim benzylhydrazon hydroxyquin- oleate	0.375-3mg /block.	Incorporated pesticides into agar blocks. Diffusion method.	*R.meliloti* *R.trifolii*	Thiram>chinon- oximbenzyl- hydrazon & hydroxy- quinoleate.	Brakel 1963
captan ceresan chloranil thiram	20,000 & 2,000 ppm solutions.	YMA. Porcelain bead/diffusion method.	*R.japonicum*	ceresan>thiram> captan>chloranil	Diatloff 1970.
ceresan phenyl- mercuryacetate cerenox-specia falisan CuSo4.5H2O ethyl-mercury silcate merkolat panogen	Not given	YMA diffusion methods.	*R.legumino* *-sarum* *R.phaseoli* *R.meliloti* *R.trifolii* *R.lupini* *R.japonicum*	Strong inhibition >50mm diam.	Keckes 1970
hexa-chloro-benzole dithane-M45 cupravit cuprosan super D		TMTD+lindane nogos 50 coprantol spergon		Medium inhibition 30-50mm diam.	
berzem zineb phygon miltox captan ziram malipur P siarcol ferbam malipur		dunakoll thiram cosan cuprox thiovit copperoxychloride delan maneb		Low inhibition 10-30 mm diam.	
captan neo-voronit	5-50ppm. 100-500 "	G:1 medium O.D in liq. media.	*R.meliloti*(2) *R.legumino* *R.trifolii*(2)	Captan inhibited at >25 micrograms. Neo-voronit inhibited at >100 micrograms.	Gillberg 1971

Fungicide	Concentration	Media/Method	Species/Strain	Effect	Reference.
antracol karathane captan ceresan wet spergon thiram dithane M45 ceredon-T ceresan rhizoctol	50,000ppm 50,000ppm 1000ppm 1000ppm 1000ppm 1000ppm 1000ppm 1000ppm 1000ppm 1000ppm	YMA. Cup-plate method.	*R.meliloti*(2) *R.trifolii*(9) *R.phaseoli*(16)	Order of toxicity; ceresan wet>thiram & ceredon-T>thizoctol & ceresan>captan & dithane-M45>spergon antracol>karathane	Fawaz et al.1972
benomyl captan triforine thiophanate-methyl carbendazim carboxin dodine dimethirimol ethirimol ethylan CP oxycarboxin thiram tridemorph	10-200ppm	YMA. Pesticide incorporated into media. Colony growth measured.	*R.trifolii*	Captan, dodine & oxycarboxin caused reduction in growth.	Fisher 1976
thiram	0.1-10X recommended levels.	YMA. Diffusion methods.	*R.japonicum*	Inhibited growth at all concentration	Tu 1977.
manoxol OT alk-3 triton X45 PP 222 ethylan CP hyamine 3500 aromox DMMCDW aromox C12W	50-100 ppm. 100-1000 " 50-200 " 100-1000 " 100-500 "	YMA. Pesticide incorporated into media. Colony 5-10 " 10-80 " 20-80 "	*R.trifolii* growth recorded.	order of toxicity; hyamine 3500> manoxolOT= aromox DMMCDWW & C12W>alk3,PP222 & triton X45.	Fisher et al. 1978.
triademefon	50-1000ppm	YMA. Pesticide incorporated in the media. Colony growth recorded.	*R.trifolii*	50mg/l had no effect.1000mg/l caused 15% inhibition.	Fisher et al. 1979.
actidione benlate captan dithane SPC fernasan karathane thiram	420 ppm 3000 ppm 2000 ppm 5000 ppm 1500 ppm 5000 ppm 4000 ppm	YMA diffusion methods	19 rhizobial strains isolated from 9 legume hosts.	Order of toxicity;dithane-M45,karathane> captan & fernasan> actidione & thiram> benlate.	Faizah et al. 1980.

Fungicide	Concentration	Media/Method	Species/Strain	Effect	Reference.
benomyl captan carbendazim chloroneb chlorothalonil dodine fenaminsulf folpet metazoxolon pyroxychlor quintozene thiram zineb	5-5000ppm	YMA. Diffusion methods.	*R.japonicum*(3)	Order of toxicity;thiram> captan,zineb> chlorothalonil,dodine folpet & benomyl, fenaminsulf,chloroneb, >carbendazim,pyroxy-chlor,quintozene & metazoloxon.	Tu 1980
benodanil benomyl carboxim ethirimol fenarimol oxycarboxin pyracarbolid tridemorph triforine	2-15 ppm 10-50 ppm 10-50 ppm 10-50 ppm 0.5-5 ppm 10-50 ppm 0.5-5 ppm 2-15 ppm 1-5 ppm	YMA. Pesticides incorp-orated into media. Colony diameter measured.	*R.trifoli*	Only fenarimol & oxycarboxin affected growth at these levels.	Fisher & Hayes. 1981.
metalaxyl benalaxyl iprodione captafol	2000 ppm solution	YMA. Porcelain bead method.	*R.japonicum*	No effect	Diatloff 1986.

Faizah *et al.*(1980) treated 19 strains of 9 species of rhizobium with 7 fungicides. The order of toxicity obtained was dithane M45 and karathane WD >> fernasan > captan > thiram > actidione > benlate, once again captan was ranked as more toxic than thiram. Fisher *et al.*(1978) measured O_2 uptake and increase in colony diameter of *R.trifolii* grown for 35 days on media with fungicide incorporated. Hyamine 3500, triton X45 and ethylan CP were very toxic. Alk3 and PP200 were less toxic. Fisher *et al.*(1979) found only concentrations of triademefon above 500ppm reduced radial growth of *R.trifolii* colonies by 50 %.

4.4. Insecticides.

(Refer Table 3.)

Wilson and Choudri (1946) applied DDT to 11 rhizobial species isolated from legume nodules. They recorded that agar was covered with copious rhizobial growth after 10 days.Kapusta and Rouwenhurst (1973) applied 9 different insecticides to *R.japonicum*. Disulfoton, an organic phosphate insecticide was found to be bacteriostatic at recommended levels. Carbaryl exhibited some inhibitory activity against some strains. Aldrin, azinphosmethyl, dasanit, diazinon, dieldrin and lindane were all found to be non-toxic at levels of 1 to 10 times the recommended rate. Three insecticides were tested on 10 strains of *R.japonicum* (Mallik and Tesfai 1983). Diazinon was found to be non-toxic, carbaryl was only toxic at high levels and malathion was toxic at all levels above 25 ppm which disagrees with the results of Kapusta and Rouwenhurst (1973) who recorded malathion as non toxic at 15-150ppm and Brockwell and Robinson (1976) who found malathion, phosmet, methidathion, dimethoate and formothion exhibited no effect on 3 species of rhizobia at 1000ppm.

Funke,Naik and Schulz (1969) found 3 organophosphorous insecticides varied in their effects toward rhizobia when applied at levels ranging from 1 to 100 times the recommended level. Dasanit inhibited *R.japonicum*, *R.leguminosarum*, *R.meliloti and R.trifolii*, whereas diazinon had no effect on any of these, which is in agreement with the results of Mallik and Tesfai (1983) and Kapusta and Rouwenhurst (1973) for this insecticide. Diatloff (1970) absorbed insecticides onto hollow porcelain beads and ranked insecticide toxicities based on inhibition zones as dimethoate > lindane > isobenzan > endrin > dieldrin. This author found wet beads gave greater inhibition zones than dry beads.

R.japonicum was treated with insecticides at 0.1 to 10 times the field rates *in vitro*. Chlorpyrifos caused a small inhibition at 0.1 to 1 times, and considerable inhibition at 10 times rates. Lindane exhibited moderate toxicity at 1 and 10 times levels (Tu 1977). Brakel (1963) found lindane applied in small agar blocks at the higher rate of 1500 ppm was inhibitory to growth of 4 rhizobium species. Aldrin showed a slight toxicity at 3000 ppm while parathion showed no effect. However these levels are very much higher than those that could be reasonably expected to be reached in the soil.

Table 3.
Insecticide Effects on Rhizobia.

The accepted common name as given in The Pesticide Manual of the British Crop Protection Council 6th ed.(1979) is used wherever possible.

If no common name is available the chemical name, trivial name or trade name is used.

Insecticide	Concentration	Media/Method	Species/Strain	Effect	Reference
DDT	not given	Agar-media-pesticide incorporated into the media.	11 strains isolated from legume hosts.	Copious growth after 10 days ie.no effect.	Wilson & Choudri. 1946
lindane aldrin parathion	0.375-3mg/block	Incorporated into agar blocks. Diffusion methods	*R.meliloti* *R.trifolii*	Order of toxicity; lindane>aldrin>	Brakel 1963 parathion.
dasanit diazinon phorate	1-100X recommend-ed levels.	Nutrient agar Diffusion methods	*R.japonicum* *R.legumino-sarum* *R.meliloti* *R.trifolii*	Order of toxicity;-dasanit>phorate >diazinon.	Funke 1969.
lindane endrin dieldrin dimethoate isobenzan	2000 & 20,000 ppm solutions	YMA,porc-elain beads. Diffusion methods.	*R.japonicum*	Order of tox-icity:-dimethoate >lindane>isobenzan> endrin>dieldrin.	Diatloff 1970
trichlorofon phosphonate trichloronate carbophenothion dursban nemacur P aprocarb carbofuran aldicarb	100% in acetone.	YMA, diffusion methods.	*R.meliloti* *R.leguminosarum* *R.trifolii* *R.japonicum*	Order of toxicity;-trichlorfon & trichloronate> aprocarb & aldicarb> nemacur P>carbophen-othion>dyfonate & dursban.	Shin-Chsiang Lin et al. 1972.
aldrin azinphosmethyl carbaryl dasanit diazinon dieldrin disulfoton lindane malathion methoxychlor	25ppm 12.5ppm 70ppm 2.5ppm 25ppm 7.5ppm 20ppm 15ppm 2.5ppm	YMA. 15ppm plate technique.	*R.japonicum* Gradient	Disulfoton growth. None of the other pesticides had any effect.	Kapusta inhibited & Rouwen-hurst. 1973.

Insecticide	Concentration	Media/Method	Species/Strain	Effect	Reference
malathion phosmet Robinson methidathion dimethoate formothion	Undiluted & diluted to 10,000ppm	YMA diffusion methods.	*R.japonicum* *R.meliloti* *R.trifolii*	Order of toxicity;- formothion> dimethoate>methid-athion>malathion & phosmet.	Brockwell & 1975
lindane chlorpyrifos	0.1-1 x recommended levels	YMA diffusion methods.	*R.japonicum*	Lindane was moderately toxic at 10x.chlor-pyrifos slightly inhibited at both levels	Tu 1977
aldicarb	0.2-10ppm	Malt extract media. respiration & O.D.measured.	Cowpea Rhizobial	2ppm stimulated growth.5 & 10ppm inhibited growth.	Sekar & Balasbru-mamanian 1979
aldrin heptachlor rogor 40	500 ppm 52 ppm 37,800 ppm	YMA diffusion methods.	19 rhizobia isolated from 9 hosts.	Order of tox-icity;rogor 40> aldrin>heptachlor.	Faizah et al. 1980.
carbaryl diazinon malathion	0.5-5 X rec-ommended levels. (=5-250 micro-grams/disk.)	YMA	*R.japonicum*(10) diffusion methods.	Order of tox-icity;malathion >carbaryl> diazinon.	Mallik & Tesfai 1983.

Lin *et al.*(1972) tested 9 insecticides for effects on growth of 4 species of rhizobial bacteria. Dyfonate and dursban had no effect on any of the rhizobia at very high concentrations (table 3). Carbophenothion caused a slight inhibition of *R.trifolii* at 20 microlitres/disc. The most toxic were trichorofon and trichloronate, followed by aprocarb and aldicarb and then by nemacur-P. However aldicarb was found to stimulate growth of a cowpea rhizobia at the low level of 2ppm, and slightly inhibit growth at 5 and 10 ppm (Sekar and Balasbrumanian 1979).

4.5. Concluding comments.

Toxicity of pesticides toward rhizobia does not generally appear to be linked to a particular group of pesticides or a specific type of compound. For this reason it is extremely difficult to extrapolate results or make predictions on the toxicity of an untested pesticide. Hence all new products which may be used in situations leading to contact with rhizobia require tested on rhizobial bacteria and symbiotic legumes.

Diazinon, dimethoate and malathion are 3 insecticides for which contradictory results have been obtained. There are other examples of similar variation in the literature which may often be due to variation in experimental technique, materials or strains as discussed previously. Reasons for such contradictions are often not clear due to the lack of experimental detail in published reports. It is these discrepancies which highlight the fact that more research is needed.

Chapter 5.0. Toxicity testing of Pesticides on Legume-Rhizobium Symbioses - Concepts.

5.1. Preamble.

Reports of toxicity of pesticides toward legume growth and, in particular, legume nodulation vary considerably. Further research is necessary to elucidate variation in effect and to ascertain whether it arises from differences in soil type and conditions, or in part at least, from experimental techniques.The reasons for variation in results can be classified into 5 categories. These are;

(i)Species/strain variation.

(ii)Conditions of the experiment (field or laboratory).

(iii)Timing of pesticide applications.

(iv)Method of application.

(v)Parameters used to measure toxicity.

5.2.Variation due to species or strain.

Toxic effects of a pesticide can vary widely between plant species and cultivars. *Trifolium pratense* (red clover) was less affected by increasing pesticide concentration than *T.repens* (white clover)or *T.dubium* (small hop clover) (Brock 1972). Funke *et al.*(1969) sprayed dasanit, diazinon and phorate at levels from 1 to 100 times the field rate on *Medicago sativa* (alfalfa), *Melilotus alba* (sweetclover) and *Glycine max* (soybean). *G.max* was affected only at the 100 times rate. *M.alba* was affected by high concentrations of dasanit and phorate causing lowered nodulation and growth. *M.sativa* was sensitive to all insecticides tested. Smith *et al.*(1978) found two varieties of *M.sativa* showed differing sensitivities. *T.pratense* and *Melilotus alba* were damaged more than *M.sativa* at the same concentrations. However Lin *et al.*(1972) found sensitivity of *T.pratense* and *M.sativa* to pesticides was very similar, as was sensitivity of *Vicia faba* and *Lens esculenta*. Greaves *et al.*(1978) attempted to achieve uniform plant growth by careful thinning but found by 7 weeks growth, shoot fresh weights varied by a factor of 2 in control stands. Hence the lack of uniformity of plant growth, even within one strain is quite large, and must be allowed for when planning an experiment.

The variety of results emphasize the importance of testing on all plants that may be contacted. Results for one crop species cannot be extrapolated to another. Even results from one crop species can vary dependant on the cultivar and other interacting factors.

5.3.Field versus laboratory trials.

Pesticides are applied mainly in agricultural environments hence toxicity testing should ideally be carried out under similar conditions. It is recognized by all researchers that simplified systems do not completely reflect the actual environment. However the inherent variability of natural systems has meant researchers need to simplify test situations to isolate cause and effect mechanisms.

Appleman and Sears (1946) found legumes grown in soil did not develop symptoms of pesticide injury to the same degree as those grown in sand. This may be due to the adsorption of pesticides to soil colloids. In contrast Lindstrom *et al.* (1985) found dinoseb exhibited the same effect on nitrogenase of legumes in sand/vermiculite culture as in the field. Slight differences in yield were noted which the authors attributed to mechanical damage due to hand-weeding of field plots. Smith *et al.* (1978) found significant differences in nitrogenase activity between soil and non-soil substrates in tests for plant-pesticide interactions. Organo-phosphorous and carbamate insecticides were applied at 5 and 50 ppm to legumes grown in soil and vermiculite. Alfalfa, sweetclover and red clover were damaged by the carbamates (carbaryl, carbofuran and aldicarb) in soil and by the organophosphates (terbufos, phosmet, oftanol and chlorpyrifos) in vermiculite. Garcia and Jordan(1969) reported that 2,4-D had a more severe effect on nodulation in growth chamber experiments using sterile soil, than in the field.

Dunigan *et al.*(1972) found pesticides exhibited no effect in field trials on legumes, but toxic effects were detected in glasshouse tests dependent on soil type. The possibility that soil microflora can detoxify a chemical may also be a major factor in activity of pesticides in the soil. Hence the growth medium used should attempt to simulate as much as possible the agricultural soil in which the crop is grown.

5.4.Timing of pesticide application.

The timing of pesticide application may markedly affect the plants response. Timing of application of trifluralin was found to be crucial. Nodulation of *Vigna unguiculata* was stimulated by a pre-emergent application, but application at planting inhibited nodulation (Hamdi and Tewfik 1969). Timing of pesticide applications during toxicity testing should reflect the manufacturers recommendations.

5.5.Method of application.

The method of application of a pesticide may also have an effect on the degree of toxicity. Peters and Ben Zbiba (1979) suggested that pesticides applied to the rooting media may be expected to have more influence on nodulation than those applied to foliage. However Lindstrom *et al.*(1985) found foliarly applied dinoseb at recommended levels suppressed nitrogenase activity while soil application had no effect. It was thought the suppression was indirect through interference with energy metabolism via photsynthesis.

Johnen *et al.*(1978) attempted to standardize conditions for toxicity tests regardless of the use pattern and properties of the test chemical. These authors concluded that uniform experimental conditions could not be used when studying pesticidal effects on symbiotic nitrogen fixation. Due account must be made of the nature and useage of the chemical if the real effects of pesticides are to be studied.

5.6.Parameters of toxicity.

The particular parameter measured determines the sensitivity and type of information obtained. Even when plant species used, pesticide applied and growth

conditions are identical, comparisons can be made impossible by variations in the parameters used. Parameters may range from scores of % germination and % deformation during germination (Rhys and Phung 1985) to plant height and weight (Selim *et al.*1976), leaf, stem and petiole weight (Greaves *et al.*1978) or root growth (Borbely and Keckes 1972).

Rodell *et al.*(1977) reported that organophosphorous and carbamate insecticides exhibited no effect on *G.max* growth or nitrogen fixation. In a later paper these authors stated that the acetylene reduction methods employed in the study had not been sensitive enough to detect small variations (Smith *et al.* 1978). Johnen *et al.*(1978) found the coefficients of variation were large for numbers and weights of nodules; but shoot, root and total plant weight showed much less variation. These authors conclude that nodule weight and acetylene reduction are the most reliable indications of pesticide effects on symbiotic nitrogen fixation. Plant weights and nodule numbers were also found to be useful although less reliable.

Kulkarni et al (1974) found thimet, heptachlor and dasanit caused a significant reduction in nodule numbers but an increase in individual nodule size, suggesting that the overall effect on nitrogen fixation was nil. Hamdi and Tewfik (1969) found the herbicide trifluralin inhibited dry weight and nitrogen content of plants to the same degree as nodulation. Diatloff (1983) found a positive correlation between nitrogen fixing activity and nodule numbers. Hence different parameters often indicate the same toxic effect. The selection of parameters should be as wide as possible while avoiding unnecessary duplication.

5.6.1.Acetylene reduction.

Acetylene reduction has been found by most researchers to be the most sensitive parameter to measure toxic side effects of pesticides on nitrogen fixation. The way in which acetylene reduction results are expressed can greatly bias the final interpretation. Results expressed on a whole plant basis largely reflect the size of the plant, whereas results expressed per unit weight give a more meaningful measure of nitrogenase activity.

5.7.Timing of parameter measurements.

The timing of measurements may have a critical effect on the eventual interpretation. Thiram, lindane and chlorpyrifos significantly decreased nitrogen fixation of *Glycine max* when applied as a seed dressing. This toxic effect disappeared after 6 weeks (Tu 1977). However Johnen *et al.*(1978) found acetylene reduction was markedly reduced by pesticides at 6 and 8 weeks, but the effect disappeared by 10 weeks. Fisher *et al.*(1978) found several different pesticide treatments reduced growth of *Trifolium repens* at 8 weeks growth, but after 12 weeks the effect had almost disappeared, however negative effects on nitrogen fixation were in general more pronounced at 12 weeks then at 8 weeks.

Pareek and Gaur (1970) applied DDT at levels ranging up to 1000ppm to *Phaseolus aureus.* Complete inhibition of growth occurred above 100ppm after 4 weeks

growth. However at levels below 100ppm plant growth showed signs of damage after 6 weeks exposure. The time at which a measure of toxicity is taken can be critical to the result. Consideration should be taken of the growth cycle of the test plant and the relative importance of the stage of growth to agriculture.

Chapter 6.0. Literature Review of Pesticide Toxicity towards Legumes and Nodulation.

6.1. Preamble.

Pesticides, and particularly herbicides, are applied to leguminous crops in the belief that they are ineffective against the crop plant while inhibiting growth of competing weeds. However such direct contact between a toxic agrochemical and a plant involved in a highly sensitive mutualistic symbiosis may result in toxic side-effects, hence research focuses on those pesticides regularly applied to leguminous crop plants.

Considerable research has been carried out on legume-pesticide interactions both *in vitro* and in the field. However, due to the large numbers of pesticides available, little of this research concurs, making comparisons of results very difficult. This review attempts to outline some of the studies carried out, and indicate how confused this area of research is.

6.2.Herbicides.

(Refer Table 4.)

Borbely and Keckes (1972) treated *Lupinus luteus* (lupin) in the field with 2 urease and 2 s-triazine herbicides at rates from 2 to 6 ppm. Simazine, prometryne and monolinuron all significantly reduced plant and root growth while kloben did not affect growth of this legume. Seven herbicides were found to decrease nodulation of field and glasshouse grown *Glycine max*. Prometryne, nitralin, GS16068, chloramben, trifluralin, linuron and vernolate were applied at levels recommended by the manufacturers and at 10 times these levels. Tap roots were found to be more sensitive than laterals, results generally agreed with those of Borbely and Keckes (1972) (Dunigan *et al.*1972). Kapusta and Rouwenhurst (1973) also found one to ten times the recommended rates of nitralin and chlorpropham lowered soybean fresh weight and nodulation in field trials.

When paraquat was applied to *Pisum sativum* in sand culture in the glasshouse, yield and nodulation was inhibited (Manninger *et al.*1972). However sand does not represent the field situation very realistically. Dalapon and amitrole were applied to mature lucerne *in vitro* (Lakshmi-Kumari *et al.*1974). Dalapon did not show any inhibitory effects up to 100ppm whereas amitrole severely inhibited lucerne growth. MCPB, 2,4D-Na and 2,4D-amine almost totally inhibited lucerne germination. TCA (sodium trichloroacetate) and dalapon were applied to *Pisum sativum* grown in a sandy loam soil in the glasshouse. Neither herbicide had any significant effect on the plant at 0.1mM but at 10mM adversely affected pea nodulation, dalapon was more toxic than TCA (Sud *et al.*1973). Garcia and Jordan (1969) also used a sandy loam soil in which to base field trials of dalapon and 2,4DB on *Lotus corniculatus* (birdsfoot trefoil). 2,4DB alone or in combination with dalapon caused a significant decrease in numbers of nodules and foliage dry weight. Abnormal root development was sometimes noted.

Table 4.
Herbicide Effects on Legumes.

The accepted common name as given in The Pesticide Manual of the British Crop Protection Council 6th ed.(1979) is used wherever possible.
If no common name is available the chemical name, trivial name or trade name is used.

Herbicide	Concentration	Media/Method	Species/Strain	Effect	Reference
Phenoxyacetics 2,4D MCPA Phenoxybutyrics. MCPB 2,4-DB	0.01-5ppm *post-em.	*In vitro* Mineral agar	*Trifolium repens* (white clover)	Phenoxyacetics more toxic then phenoxybutyrics.	Fletcher et al. 1956
paraquat	0.34-1.36 lb/acre. post-em.	Field trials. Soil type not stated.	*T.repens.*	Had no lasting effect on yield or seed viability.	Blood 1962
2,4DB dalapon	1.26k/ha. 4.48k/ha. post-em.	Field trials & growth chamber experiments in a sandy loam soil.	*Lotus corniculatus* (birdsfoot trefoil)	2,4DB sig. decreased nodule numbers & foliage dry weight. Dalapon was less toxic.	Garcia & Jordan 1969.
kloben aresin prometryne prometryne+ simazine	4.5-5.9 k/ha. 2.0-3.5 k/ha. " " " " post-em.	Field trials Slightly acidic brown forest soil.	*Lupinus luteus* (sweet lupin)	Kloben did not inhibit root weight & yield. All other herbicides did.	Borbely & Keckes 1972.
chloramben GS16068 linuron nitralin prometryne trifluralin vernolate	0.56-14k/ha. 0.34-8.4k/ha. 0.22-5.6k/ha. 0.16-4.2k/ha. 0.22-5.6k/ha. 0.16-4.2k/ha. 0.56-14k/ha. pre-em.	Field & pot trials in 1 loam & 4 silt loams.	*Glycine max* (soybean)	No real detrimental effects. Tap root nodules more sensitive then lateral root nodules.	Dunigan 1972.
trifluralin carbetamide	1.0 k/ha a.i. 2.0 k/ha.a.i. pre-em.	Field trials loamy sand soil.	*Trifolium pratense* (broad red clover) *Trifolium repens* *Trifolium dubium* (suckling clover) *Lotus pedunculatus*	Both herbicides decreased nodule numbers and plant fresh weight.	Brock 1972
paraquat	16mg/pot pre-em.	Pot trials soil type not stated.	*Pisum sativum* (pea)	Depressed yield and nodule number.	Manninger *et al.* 1972.
chlor-propham nitralin	3.4-34k/ha. 1.1-11.2k/ha. post-em.	Field trials in a silt loam soil.	*Glycine max*	Lowered plant (soybean) & nodulation.	Kapusta fresh weight Rouwenhurst 1973.

Herbicide	Concentration	Media/Method	Species/Strain	Effect	Reference.
TCA dalapon	0.1-10mM pre-em.	Pot trials in a sandy loam.	*Pisum sativum* (pea)	No effect at 0.1mM.Toxic above this level. Dalapon>TCA#.	Sud *et al.* 1973.
MCPB paraquat 2,4D dalapon amitrole	50-1000micro-grams/ml. pre-em. & post-em.	*In vitro* Jensens N_2 free agar.	*Medicago sativa* (lucerne)	MCPB almost totally inhibited germination. Amitrole>dalapon on plant growth.	Lakshmi -Kumari 1974.
benefin profluralin diclofop -methyl EPTC	1.12-1.68k/ha. " " " pre & post-em.	Pot trials in a silt loam & a sand.	*M.sativa* *T.pratense*	Benefin & profluralin reduced growth. Diclofopmethyl reduced N-fixation.	Peters & Ben-Zbiba. 1978.
alloxydim-sodium	2-4k/ha. post-em.	Pot trials. sandy loam.	*P.sativum.* (pea)	Significant reduction in growth and nodulation.	Greaves *et al.* 1978.
MCPA bentazone	0.2-8ppm (=0.5-1 X recomm-ended levels)	*In vitro* & in soil. Soil type not stated.	*Trifolium pratense* (red clover)	Deformation of root hairs & ineffective nodule formation.	Ljunggren & Mart-ensson. 1980
alachlor metribuzin trifluralin glyphosate 2,4-D	1-5 * recomm- 7d pre-em. or 17d post-em.	Pot trials in a sandy loam soil.	*Glycine max* (soybean)	Pre-em. reductions in nod-ules & N-content. post-em.metribuzin, trifluralin decreased plant growth.glyphosate decreased nodulation	Mallik & Tesfai. 1985.
22DPA fusilade TCA trifluralin EPTC carbetamide sethoxydim propyzamide	0.06-9.0 micrograms /seed.	*In vitro*	*Trifolium repens* (white clover)	Only 22DPA & fusilade did not inhibit germin -ation.22DPA red -uced germination and plant dry weight.	Rhys & Phung. 1985.

* - post-em. refers to pesticide application after germination of the crop.
pre-em. refers to pesticide application before germination of the crop.
- > stands for "greater than" and is used here to denote a ranking of toxicity.

Benefin, profluralin, diclofopmethyl and EPTC were sprayed at recommended levels onto alfalfa and red clover growing in a silt loam in a glasshouse. Benefin and profluralin decreased height and weight of plants and also damaged alfalfa nodulation. Diclofopmethyl significantly reduced nitrogen fixation of both plant types but had no effect on root weight and nodule number. It was suggested that reduction in nodulation may be due to less root surface area available for infection (Peters and Ben Zbiba 1978). A variety of herbicides were tested to find which best controlled grass competition in clover:grass swards. Only 22DPA (75% dalapon) and fusilade did not cause deformation of clover seedlings. However these herbicides did not increase clover growth in the field. It was concluded that either the grass was not competing with the clover, or the herbicides were damaging clover growth (Rhys and Phung 1985).

Hormone herbicides were applied to white clover *in vitro* at concentrations ranging from .05 to 5ppm. Phenoxyacetics were shown to be toxic whereas Phenoxybutyrics were not (Fletcher *et al.*1956). MCPA in root media at 1/2 and 1 times recommended levels caused alterations of the entire development of the root system of *Trifolium pratense*. Bentazon had a mild effect up to 8ppm, lowering numbers of root hairs infected. Normal rates of both, foliarly applied, caused deformation of root hairs in soil and ineffective nodule formation (Ljunggren and Martensson 1980). Mallik and Tesfai (1985) treated soybean with alachlor, metribuzin, trifluralin, glyphosate and 2,4DB at field rates and 5 times these. All herbicides applied pre-emergence (ie. before crop planting) significantly reduced nodulation, acetylene reduction and nitrogen content. When applied post-emergence alachlor did not affect soybean growth but metribuzin did. Trifluralin applied to soybeans grown in a sandy loam soil in a greenhouse significantly reduced leaf, stem and petiole weight although root growth was little affected. However Dunigan (1972) found trifluralin did not affect growth of soybean at recommended concentrations.

6.3. Fungicides.

(Refer Table 5.)

Fungicides on crops are often applied in the form of seed protectants. Researchers have therefore attempted to simulate this application method during experiments of fungicide toxicity to legumes. Fungicide seed dressings of ceresan and thiuram decreased N and P content of *Vicia sativa* but were found not to affect nodulation (Elek and Keckes 1972). However Taha *et al.*(1972) found ceresan at 0.5% per seed weight decreased nodulation, dry weight and N content of *Vicia faba* and *Lens esculenta*. This variation in result may be due to differences in concentrations of fungicide applied, however levels applied were not stated by Elek and Keckes. Ceresan, thiram, captan and chloranil applied as seed dressings at 0.2% per seed weight were found to cause a slight reduction in nodulation of soybean (Diatloff 1970), which agrees with the result of Taha *et al.*(1972).

Different fungicides exhibited variable effects when applied as seed dressings to *Vigna anguiculata* (ground nut) grown in quartz sand. Brassicol, terracoat

Table 5.
Fungicide Effects on Legumes.

The accepted common name as given in The Pesticide Manual of the British Crop Protection Council 6th ed.(1979) is used wherever possible.
If no common name is available the chemical name, trivial name or trade name is used.

fungicide	Concentration	Media/Method	Species/Strain	Effect	Reference
ceresan thiram captan chloranil	0.2% w/w of seed.	Field trials. Sandy loam & sandy soils.	*Glycine max* (soybean)	Slight reduction in nodulation of tap-roots.	Diatloff 1970.
panogen phygon captan ceresan copperoxy-chloride	1.4g/kg seed 1.2 " 2.0 " 2.0 " 2.0 "	*In vitro*,sand & soil.Applied as seed dressings.	*Vicia sativa* (vetch)	Order of toxicity; ceresan>panogen& thiram>captan> phygon>spergon= copperoxychloride.	Keckes 1970.
ceresan thiram	Not given	Field trials in sandy soil. Applied as seed dressings.	*V.sativa*	No effect on nodulation. Incresed yield & protein content.	Elek & Keckes 1972.
ceresan	0.5 % per seed weight.	Pot trials in clay loam soil.Applied as seed dressings.	*Vicia faba* (bean) *Lens esculenta* (lentil)	Decreased nodulation,dry weight & N content.	Taha *et al.* 1972.
brassicol terracoat thiram benlate botran-difolatan 50:50 captan dexon dithane SPC manzate saadtan vitavax	2g/kg	Pot trials. Quartz sand. Pesticides applied as seed dressings.	*Vigna unguiculata* (Ground nut)	Brassicol,terracoat & thiram had little toxicity. Others all either inhibited nodulation or stunted tap root development.	Staphorst & Strijdom 1974.
oxycarboxin dimethirimol dodine captan carbendazim ethylan CP thiram ethirimol	250-500ppm " " " " " " "	Pot trials. Potting soil. Pesticides were applied post-em.	*Trifolium repens* (white clover)	Oxycarboxin & dimethirimol were toxic at 250ppm.Others had no effect up to 500ppm. Ethylan CP & thiram at 250ppm stimulated N-fixation.	Fisher 1976.
manoxol OT triton X45 PP222 alk 3	1000-5000 micrograms per gram of soil.	Pot trials Potting soil. Fungicides applied post-em. to soil surface.	*T.repens*	Only high levels(above recommended levels) decreased plant weights.	Fisher *et al.* 1978.

Fungicide	Concentration	Media/Method	Species/Strain	Effect	Reference.
triademefon	0.5-50mg/k	Pot trials. potting soil.	*T.repens*	Only levels above those recommended lowered weight & N-fixation.	Fisher *et al.* 1979.
fernasan captan furaden	not given	Leonard jars. sand.Fungicides applied as seed dressings	*Centrosema pubescens*	Fernasan decreased growth & nodulation. Others had no effect.	Faizah *et al.* 1980.
benodanil benomyl carboxin ethirimol fenarimol oxycarboxin pyracarbolid tridemorph triforine	2-50mg/kg Levels based on estimate of likely amount to accumulate over a year.	Pot trials. Potting soil. Fungicides soil applied post-em.	*T.repens*	Recommended levels had no effect.High levels of carboxin, oxycarboxin,benodanil, pyracarbolid & tridemorph affected plant weights.	Fisher & Hayes. 1981.
carboxin captan PCNB	1-10 X recommended levels.	Field trials. sandy loam soil.Fungicides were applied as seed dressings.	*Glycine max.* (soybean)	recommended levels had no effect.Higher levels caused some inhibition of nodulation & growth.	Mallik & Tesfai 1985.

and thiram showed little toxicity. Others (benlate, botran-difolatan 50:50, captan, dexon, dithane SPC, manzate, saadtan and vitavax) all either impaired or inhibited nodulation. All tended to stunt tap root development (Staphorst and Strijdom 1976). Keckes (1970) also found variable results when a range of fungicides were applied at recommended levels to *Vicia sativa*. Effects ranged from inhibition of nodulation and decreased yield, to no effect. The fungicides were ranked on toxicity in the order ceresan > thiram > captan > phygon > spergon=copperoxychlor.

Mallik and Tesfai (1985) found carboxin, captan and PCNB at recommended levels did not affect plant growth, nodulation, nitrogen fixation or total N content of *Glycine max* (soybean). Higher than recommended rates did cause some deleterious effects. Tap root nodulation was particularly affected, as was found to occur by Staphorst and Strijdom (1976) and Dunigan *et al.* (1972) when *Glycine max* was treated with herbicides (section 6.2.). None of the fungicides tested by Fisher (1976) showed any inhibition of white clover growth or nitrogen accumulation at recommended levels. Oxycarboxin and dimethirimol were toxic at 250ppm in soil whereas dodine, captan, carbendazin, ethylan CP, thiram and ethirimol caused no significant difference from the control at 500ppm, a much higher level of captan then that recorded by Staphorst and Strijdom (1976), Keckes (1970) and Mallik and Tesfai (1985) as being toxic. Fisher (1976) applied captan post emergent, whereas the other authors applied captan as a seed dressing, which may explain this difference in result. Ethylan CP at low levels (250ppm) stimulated acetylene reduction as did thiram, although oxycarboxin reduced nitrogen fixation.

Fisher and Hayes (1981) applied 9 systemic fungicides to *Trifolium repens* (white clover) plants. Recommended levels of benodanil, benomyl, carboxin, ethirimol, fenarimol, oxycarboxin, pyracarbolid, tridemorph and triforine had no effect on plant growth and acetylene reduction, in agreement with the results of Fisher (1976). Surfactant mildew eradicants had the greatest effect on nitrogenase activity of young plants (Fisher *et al.*1978). The extremely high concentration of 5000ppm of manoxol OT, triton X45 and PP222 reduced plant growth. Alk3 caused a significant increase in plant growth at all levels. Fisher *et al.*(1979) found the systemic fungicide triademefon affected plant weight and symbiotic nitrogen fixation only at levels in soil much greater than those applied in practice.

6.4. Insecticides.

(Refer Table 6.)

Wilson and Choudri (1946) found "adequate" nodulation of alfalfa, red clover, soybean and vetch after treatment with DDT at 500 to 2000ppm. However these authors offered no definition of adequate nodulation. When DDT was applied to *G.max*, *Trifolium pratense* (red clover), *Melilotus alba* (sweetclover) and lespedeza in sand culture at 11 to 1100ppm no negative effects were noted except at the highest level, at which nodulation and dry weight was significantly reduced (Appleman and Sears 1946). However this level is much greater than that likely to occur in the field.

Table 6.
Insecticide Effects on Legumes.

The accepted common name as given in The Pesticide Manual of the British Crop Protection Council 6th ed.(1979) is used wherever possible.
If no common name is available the chemical name, trivial name or trade name is used.

Insecticide	Concentration	Media/Method	Species/Strain	Effect	Reference
DDT	500-2000ppm	Field trials. Fine sandy loam. DDT was incorporated into the soil.	*Medicago sativa* (alfalfa) *Trifolium dubium* (red clover) *Glycine max* (soybean) *Vicia sativa* (vetch)	Recorded nodulation as being adequate.	Wilson & Choudri 1946.
DDT	11-1100ppm	Pot trials. Sand.DDT was incorporated into the media.	*Trifolium repens* (white clover) *T.dubium* *G.max*	No effect except at the highest level where dry weight & nodulation of plants was reduced.	Appleman & Sears 1946.
phorate diazinon dasanit	1-100X recommended levels.	Pot trials. Soil type not stated. Insecticides applied post-em.	*M.sativa* *G.max* *Melilotus alba* (sweet clover)	Phorate inhibited nodulation. Diazinon & dasanit inhibited plant growth only at the highest level.	Funke *et al.* 1969.
dieldrin lindane	50-1000ppm	Pot trials. Clay soil. Insecticides incorporated into medium pre-em.	*G.max*	50ppm inhibited flowering.Higher levels inhibited plant weight & height.	Selim et al. 1970
dursban aprocarb carbofuran aldicarb trichlorofon phosphorate trichloronate carbophenothion nomacur-P.	50-500ppm	*In vitro.* Plants grown on agar.	*M.sativa* *Melilotus alba* (sweet clover)	Dursban,aprocarb carbofuran & aldicarb had no effect up to 50ppm.Above this they severely decreased plant dry weights. Others had no effect up to 500ppm.	Lin 1972.
dipterex endrin	1.5k/feddan 2.0k/feddan	Pot trials. Clay loam soil.Post-em.	*Vicia faba* (bean) *Lens esculenta.*(lentil).	Dipterex increased nodulation, dry weight & nitrogen content.Endrin decreased all these parameters.	Taha *et al.* 1972.
disulfoton carbaryl	1.7-17k/ha. 2.8-28k/ha. (1-10X recommended levels).	Field trials. Insecticides surface applied post-em.	*G.max*	no effect on yield or nodule numbers.	Kapusta & Rouwenhurst 1973.

Insecticide	Concentration	Media/Method	Species/Strain	Effect	Reference.
formothion phosmet malathion methidathion dimethoate	17-700ppm (1-10X rec- omended levels).	*In vitro* Grown on Jensens media. Foliarly applied.	*M.sativa* *Trifolium* *subterraneum* (subterraneum clover.	None affected N- fixation.Highest levels inhibited plant growth.	Brockwell & Robin- son. 1976.
dinoseb acetate permethrin	2.6-26 k. a.i./ha. 0.25-2.5 k. a.i./ha.	Pot trials. Loamy sand & coarse sand. Pre & post-em.	*G.max*	Permethrin reduced growth & nodulation Dinoseb-acetate had no effect.	Johnen *et al.* 1978.
aldicarb	10ppm	Pot trials Garden soil.- Pre-em. applic- ation.	*Vigna unguic-* *ulata.* (cowpea)	Reduced nodule number,plant growth & N content.	Sekar & Balas- brumaman- ian. 1979.
aldrin	Not given.	Leonard jars. Quartz sand. Applied as seed dressing.	*Centrosema* *pubescens*	Significantly reduced nodule numbers.	Faizah *et al.* 1980.
acephate carbaryl diazinon malathion	1-10X recomm- ended levels.	Pot trials. Sandy loam soil. Pre-em. application.	*G.max*	Some inhibition of N-fixation at 100X levels. Stimulated shoot & root growth at up to 10x levels.	Mallik & Tesfai 1985.

Herbicide activity in sand culture also would be expected to be markedly different from that in soil.

Dieldrin and lindane at recommended levels (50-60ppm) and 20 times these were applied to soybean plants. Recommended levels had no effect on the plant growth but did inhibit flowering. Higher concentrations inhibited plant height and weight (Selim and Mahmoud 1970). Brockwell and Robinson (1976) treated lucerne and subterranean clover with 5 organophosphorus insecticides (formothion, phosmet, malathion, methidathion and dimethoate.) at recommended (17-70ppm) rates and ten times these *in vitro*. No evidence was found of any influence on nitrogen fixation although the higher levels did inhibit plant growth.

Sekar and Balasbrumanian (1979) treated cowpea with aldicarb at 10ppm in a garden soil in the glasshouse. Nodule number, plant growth and nitrogen content were significantly reduced. Dursban (an Organophosphate), aprocarb, carbofuran and aldicarb (Carbamates) at 500 ppm severely decreased dry weights of sweetclover and alfalfa *in vitro* whereas at 50ppm there was no toxic effect. This is a markedly different result for aldicarb from that found by Sekar and Balasbrumanian (1979). Lin carried out experiments *in vitro* while Sekar and Balasbrumanian experimented in field soil, therefore the difference in result is probably due to the different methods used.

Acephate, carbaryl, diazinon, malathion and toxaphene were found to stimulate shoot and root growth of soybean at 1 to 10 x the recommended levels. However some inhibition of nitrogen fixation and N content was found at the 100 times level (Mallik and Tesfai 1985). Funke *et al.* (1969) found phorate inhibited nodulation of 3 legumes at levels of 1 to 100 times that recommended. Plants were stunted and chlorosis observed. Diazinon and dasanit inhibited growth of alfalfa at the 100 times level only. Dipterex at 3ppm increased the number of nodules, dry weight and N content of broad beans and lentils, while endrin at 1ppm did the exact opposite (Taha *et al.*1972).

Dinoseb acetate and permethrin at field rates were applied to soybeans in various soil types in pots. Dinoseb acetate did not decrease any of the growth or nodulation parameters, however permethrin reduced shoot, nodule and total plant weight and nitrogen fixation by 6-8 weeks growth (Johnen *et al.*1978). Aldrin applied as a seed dressing reduced dry matter accumulation of *Centrosema Pubescens* but not significantly. However nodule numbers were significantly lower in treated plants (Faizah *et al.*1980).

6.5. Concluding comments.

Most of the research carried out on pesticide side-effects toward legumes are simple trials, often of very high concentrations of the pesticides, or under conditions unlikely to occur in practice. Little of the research has been expanded, and many authors have examined a similar aspect several times, hence progress in this field has been slow and uneven. To determine the true effect of pesticides on legume-rhizobium symbioses a more pragmatic approach is required. This thesis examines this toxicity problem from several approaches in an attempt to provide a clearer view of the problems involved and the probable effects of pesticides applied to legume crops.

Chapter 7.0. Study Aims.

Two of the most important types of agricultural crops are cereals and legumes. The productivity of forage and grain legumes is contributed to directly by nitrogen fixation, the importance of this to agriculture cannot be over-emphasized.

The objective of this study is to determine if the application of herbicides used commonly on white clover seed crops have any effect on;-

(i)the growth of the free-living bacteria *Rhizobium trifolii*, which forms a symbiotic association with white clover,

(ii)the growth and development of the legume *Trifolium repens* (white clover),

and (iii)the effectiveness of the symbiosis which is commonly formed between these two participants.

The experiments were designed to approach these problems using *in vitro* and *in vivo* experiments in order to establish the reliability of an *in vitro* situation in determining possible field toxicity. *In vitro* experimentation allows greater control over growth parameters. Interactions of supplied nitrogen, rhizobial inoculation, concentration and time of herbicide application may be therefore be followed in detail. *In vivo* experiments in pots within a glasshouse provide a step between in *vitro* experimentation and the field situation while indicating whether conclusions drawn from *in vitro* experiments provide an accurate assessment of effects that may occur in the natural environment.

Electron microscopy of nodules collected from plants treated *in vitro* is used to determine whether herbicides exert a direct activity on nodule functioning, and if so, where this activity is targeted.

Chapter 8.0. Methods and Materials.

8.1. Herbicides used.

The herbicides used were selected for study on the basis of their use in New Zealand white clover seed crops. All herbicides were used in their commercial formulations.

Paraquat (1,1'-dimethyl-4,4'-bipyridylium ion) (obtained from Ivon Watkins-Dow Ltd.) is a general contact herbicide used as a grass suppressant in clover crops to increase seed yields (Haggar 1974).

MCPB (4-(2- Methyl-4-chlorophenoxy)butyric acid) (obtained from Ivon Watkins-Dow Ltd.) is used for selective control of broad leaved weeds (IWD technical communication).

Bentazone (3-isopropyl-1H-2,1,3-benzothiadiazin-4(3H)-one,2,2-dioxide) (obtained from BASF New Zealand Ltd.) is a contact broadleaf herbicide used for control of chamomiles, spurrey and may weeds, post emergent (Drescher & Otto 1972).

Fusilade (butyl 2-[4-(6-trifluoromethyl-2-pyridyloxy) phenoxy] propionate) (obtained from ICI Tasman Ltd.) is believed to be only effective on the Graminaceae. This herbicide controls a wide range of annual grasses and perennial grasses from fragmented storage tissues (Plowman *et al.*1980).

Kerb (3,5-dichloro-*N*-(1,1-dimethyl-2-propyl) benzamide) (obtained from Ivon Watkins-Dow Ltd.) is a broad spectrum herbicide effective in control of annual grasses and several broad leaf weeds (Fisher 1974)

8.2. *In vitro* testing.

The herbicides selected for study were not all soluble in water at the concentrations required. Therefore, for ease of application, those non-soluble in water (fusilade and kerb) were dissolved in acetone instead of water prior to application.

Table 7. Herbicide Concentrations at 1 x recommended levels.

Herbicide.	Concentration of formulated herbicide.	Concentration of active ingredient.
Paraquat	2.5 ppm	0.5 ppm
MCPB	5 ppm	2.1 ppm
Bentazone	2.5 ppm	1.21 ppm
Fusilade	2.3 ppm	0.58 ppm
Kerb	2.02 ppm	1.01 ppm

Acetone controls were run with all toxicity tests of these herbicides to determine if this solvent had an effect on plant growth.

8.2..1.*In vitro* study of pesticide toxicity to the bacteria *Rhizobium trifolii.*

The bacterial strain used in this study was *Rhizobium trifolii* strain RS102 (ANU 9000) (provided by P.M.Gresshoff of the Australian National University). This strain effectively nodulates *Trifolium repens* (white clover), and is spectinomycin resistant (at 150 mg/l), streptomycin sensitive and kanamycin sensitive.

8.2.1.1.Herbicide toxicity toward *R.trifolii* RS102 on solid medium.

8.2.1.1.a.Methods.

All testing of herbicide toxicity toward *R.trifolii* on solid media was carried out on RGM 35 minimal media (described by Bassam & Gresshoff 1986). Stock cultures were maintained on RGM 36 complete media and modified Bergersen's minimal media (BMM) (Rolfe *et al.*1980a) (see appendix 1). The four methods of herbicide application used were;

(i)Herbicide premixed into agar prior to plate pouring.

(ii)Herbicide spread onto the surface of solid agar.

(iii)Herbicide placed in wells in seeded agar.

(iv)Filter paper discs dipped into herbicide solutions, dried and placed on to seeded agar.

It was not necessary to sterilize herbicide solutions as these compounds were so toxic that the solutions were sterile. All plates were incubated at 28^0C.

8.2.1.1.b.Seeding of agar.

Plates were seeded with *R.trifolii* RS102 by applying a dilution of a 48 hr culture to give approximately 1 X 10^2 cells per plate for methods i and ii, and approximately 1 X 10^4 cells per plate for methods iii and iv. This was considered enough to give a lawn growth of bacteria for methods iii and iv, and a resolvable number of colonies for methods i and ii. Seeding of agar for methods i and ii of necessity were carried out after herbicide incorporation into the agar. Herbicides were applied to methods iii and iv four days after the agar was seeded.

8.2.1.1.c.Herbicide application.

As commercial formulations of herbicides are used in agriculture, commercial formulations were used throughout these experiments. Herbicides were added to agar after it had been sterilized to avoid decomposition.

The concentration of herbicide incorporated into the agar in method i was based on a volume to volume ratio designed to give the same concentration as that received by the soil under a recommended spray regime assuming a soil penetration of 5cm. The amount of herbicide applied in method ii was based on a surface area ratio. Each plate received an amount equivalent to that reaching the same surface area of soil in the field. Wells in method iii were filled with a herbicide solution, which, when averaged over the volume of agar gave the equivalent concentration of that received by the soil to a depth of 5cm (Fisher and Hayes 1981). For method iv, 9mm paper discs were saturated with herbicide solutions, allowed to dry and then placed on seeded agar

plates. Herbicides were replaced with the equivalent amount of solvent to represent untreated controls.

8.2.1.1.d.Measurements.

Bacterial growth was recorded for methods i and ii by comparing the rate of colony growth to control plates. Inhibition zones about sites of application were measured in methods iii and iv. Six replicates of each treatment were made. Results were recorded after 3 days and 7 days following treatment.

8.2.1.2.Herbicide toxicity toward *R.trifolii* RS102 in liquid medium.

8.2.1.2.a. Preamble.

Growth of rhizobia in liquid culture was monitored by light density measurements on a Bausch & Lombe Spectronic 20, and by viable plate counts. Cultures were incubated in 150ml of media in 500ml flasks on a rotary shaker at 28^{o}C.

8.2.1.2.b. Herbicides.

The concentration of herbicide applied to the treatment flasks was based on the manufacturer's recommended spraying concentrations and ten times these levels. Amounts applied were calculated to be equivalent to concentrations resulting from an assumed penetration depth of 5 cm in soil (Greaves *et al.*1978;Fisher and Hayes 1981).

8.2.1.2.c. Method.

All treatments had two replicates. 150 ml of herbicide treated media was inoculated with 0.1ml of an 48hr culture of *R.trifolii* RS102. Flasks were shaken on a rotary shaker at 28^{o}C. Growth was measured at 0, 4, 24, 48 and 72 hours by densitometric methods and viable plate counts. Controls were;

(i)Acetone treatments. Controls contained dilutions of acetone equivalent to those levels of acetone received by herbicide treated flasks.

(ii)Turbidity control. A flask containing uninoculated media was used to determine if the media became more turbid during incubation.

(iii)Untreated growth controls. The herbicide treatment was replaced with an equivalent amount of water.

Viable plate counts were carried out on the same media as that contained in the test flask. The presence of any infection in the treatment flask during testing would therefore be detected. It was assumed that dilution prior to plating lowered herbicide concentrations to a level at which the herbicide would no longer affect growth of the bacteria. Any indication of possible effect of the herbicide from viable plate counts would therefore be effects that occurred in the test flask itself and not a continuing effect of the herbicide during growth on plates.

8.2.2. *In vitro* study of herbicide toxicity to *Trifolium repens* and its symbiosis with *Rhizobium trifolii* RS102.

8.2.2.1. Media.

The N free plant growth media FM (Fahreðus media) and FMNO3 (Fahreðus media with a nitrogen source) has been described by Bassam and Gresshoff (1986)(see Appendix 1). Plants were cultured in 9cm diameter petri plates using the method of Rolfe *et al.*(1980b) with slight modifications as detailed in 8.2.2.4.

8.2.2.2. Seed selection.

Procedures were carried out in order to standardize the population of seed used. It is known that the absolute number of nodules occurring per plant is dependent on plant size. However at the early seedling stage plants derived from light seed have fewer, smaller nodules than do plants from heavier seed (Mytton 1973, from Turkington & Burdon 1983).

Commercial seed of white clover (*Trifolium repens*) strain Huia S100 were sieved in order to collect the medium size range of seeds (0.84-1mm diameter). For experiments *in vitro* up to three times the number of seeds required were germinated for each group. Those with a radicle length in the middle of the distribution after 3 days germination were selected for use.

8.2.2.3. Seed sterilization and germination.

Seeds were surface sterilized with calcium hypochlorite ($Ca(OCL_2)$) prior to germination to ensure that symbioses were not established with unidentified rhizobia adhering to the seed coat. A saturated solution of $Ca(OCL_2)$ was prepared by adding approximately 1g of $Ca(OCL_2)$ to 50ml of sterile distilled water, shaken to mix and left to stand for 3 hours. Approximately 0.25g of white clover seed was soaked in sterile distilled water for 4 hours. 6 to 8ml of the saturated $Ca(OCL_2)$ solution was added to the water in which the seeds were soaking, to give an approximate 1:5 solution. Seeds were soaked for 20 - 25 minutes. The solution was then removed with a sterile pipette. Seeds were rinsed 3 times with sterile distilled water. 5 to 10 seeds were placed on nutrient media and BMM to test for contamination by soil microorganisms generally, or contaminating rhizobia specifically. Seeds were left overnight in the third soak in the dark. 20 to 30 seeds per plate were placed in rows on FMNO3. Seeds were incubated upright for 2 days at 22°C and 17 hour photoperiod.

8.2.2.4. Methods.

Seedlings were transplanted singly to FM or FMNO3, two plants per plate. Plates were left horizontal for 40 minutes after transplanting to enable seedlings to adhere to the agar. Plates were sealed with strips of Parafilm. Small slits were cut at the top to allow for gas exchange. Plates were incubated vertically in a growth room at 22°C. Light intensity in the growth room is in the order of 170 M einsteins/m^2/second which is approximately 10% of normal daylight.

Half of each set of plates (FM and FMNO3) were inoculated with *R.trifolii* RS102 after 5 days growth. 0.1ml of a 48 hr culture of rhizobia was pipetted

onto each plate between the two plants. This results in the four possible combinations of nitrogen treatment and rhizobium inoculation detailed above.

8.2.2.5. Herbicides.

Herbicide concentrations were calculated as in section 8.2.1.1.c. method ii. Herbicides were applied at two stages of plant growth. Half of the seedlings were placed onto plates already spread with herbicide at either recommended levels or ten times these. The other half of the plants received similar treatments 21 days after germination.

8.2.2.6. Controls.

All experiments included controls where herbicide applications were replaced with the same volume of solvent.

8.2.2.7. Measurements.

The group of plants treated with herbicides at 21 days were measured 3 days after germination for plantlet weight and radicle length, to determine if initial size affects rate of development within the population tested.

Preliminary experiments had shown that beyond 34 days rate of development of plants grown by this method declined. Therefore all plants were harvested 34 days after germination. By this time untreated plants grown with supplied nitrogen had reached the 4 to 5 leaf stage. Each plant was measured for shoot height, root length, fresh weight, total plant dry weight, nodule numbers, number of secondary roots, number of leaflets and nitrogenase activity by acetylene (C_2H_2) reduction assay.

8.2.2.8. Acetylene reduction.

Acetylene reduction was carried out on entire plants to determine the effect of the herbicides tested on the activity of the nitrogenase enzyme. Growth of plants on agar in petri dishes lent itself well to acetylene reduction, as the plants could be easily peeled off the surface of the agar without damage to the root system. Individual plants were placed into 29ml M^{c}Cartney bottles after bottles were flushed with N_2 gas (Masterton and Murphy 1976). Metal lids had 1mm diameter holes drilled in the centre, and were sealed with a rubber liner and aluminium foil. The rubber liner inside the cap serves to produce a seal as well as a means for injection and sampling. Aluminium foil was used to act as a barrier as ethylene is absorbed by some types of rubber (Bergersen 1980).

Controls and standards were run with each C_2H_2 reduction trial in identical chambers to the test situation, and were incubated and sampled in an identical manner (see Appendix 5 for details of method). As nitrogenase activity is believed to have a diurnal periodicity peaking at midday (Hardy *et al.*1968) all sampling was carried out between 11 a.m. and 12 a.m.

8.3.0. *In vivo* study of herbicide toxicity to *Trifolium repens* and its symbiosis with *Rhizobium trifolii* RS102.

In order to determine the degree of toxicity of the herbicides under examination to the *T.repens / R.trifolii* symbiosis in a more realistic environment, experiments were carried out on plants grown in pots in a glasshouse.

8.3.1. Soil.

A local agricultural soil was collected for glasshouse experiments in order to simulate as closely as possible a field environment. The soil was a silty clay loam, Temuka Ah. Gley (see Appendix 1 section 4 for detailed analysis). The soil was heat sterilized at 70°C for 4 hours.

8.3.2. Seed.

The seed was as used for *in vitro* experiments. Seeds were selected and sterilized as in section 8.2.2.2. and 8.2.2.3.respectively, but were not pre-germinated.

8.3.3. Preliminary experiment.

A preliminary experiment was carried out to determine possible variation in growth due to environmental variation within the glasshouse, and also to determine if the agricultural soil would function normally under a glasshouse regime.

Sixty three 9 cm pots were filled with soil to 2cm below the top. Pots were placed in a large tray filled with tap water, and left to saturate for 2 days. Approximately 50 seeds were sprinkled on to each pot, and covered with 5mm of soil. Pots were arranged in a square of 7 pots X 9 pots and watered once a day. Once seeds had germinated they were thinned to 30 per pot and inoculated with approximately 1 ml of a 48 hour culture of *R.trifolii* RS102.

Plants were harvested 34 days after seeds were planted, i.e. at the same time after germination as plants grown *in vitro*. Harvested plants were stored damp in a cold room until all determinations had been carried out. Measurements were recorded of number of plants per pot, number of nodules per pot, total dry weight and Nitrogenase activity by C_2H_2 reduction (see Appendix 4 for C_2H_2 reduction method).

Significant variation ($p<0.01$) in growth was found for number of nodules and plant dry weight among rows of pots in the preliminary trial, but not among columns, ie. plant growth was improved near the sides of the glasshouse due to more favorable environmental conditions, but did not vary from the front to the back. Hence herbicide toxicity experiments in pots were positioned in a randomized design to ensure variation due to position did not influence the outcome.

8.3.4. Toxicity testing of herbicides to *T.repens/R.trifolii in vivo.*

8.3.4.1. Method.

The soil and seed used, and the method followed was the same as for the preliminary experiment with the modifications detailed below.

Pots were filled with 350ml of sterilized soil, soaked to saturation, planted and thinned to 27 plants per pot. Pots were inoculated 5 days after germination with approximately 1ml per pot of a 48 hr culture of *R.trifolii* RS102. Pots were placed in

the glasshouse as two blocks of 8 X 8, each in a randomized design. One block was maintained a maximum water holding capacity (WHC), while the other was maintained at 50% WHC. Water holding capacity was determined by weighing pots full of dry soil then soaking them for 3 days. Soil was then allowed to drain for 6 hours and pots were re-weighed. This procedure was repeated 5 times. The resultant average % addition in weight was considered to be that required for maximum water holding capacity.

Pots were watered every day. The positions of the two blocks within the glasshouse were reversed every 3 days to minimize environmental variation between the positions.

8.3.4.2. Herbicide treatments.

Herbicide treatments were applied with a hand held pressure sprayer. The volume of liquid the sprayer expelled per minute was calculated. Herbicide levels applied were calculated based on those recommended by the manufacturers per unit surface area and at 10 times these levels.ie;

$$\text{Amount of herbicide/pot} = \text{Amount of herbicide/ha.} \times \frac{\text{Area of pot (cm2)}}{\text{Area of 1 hectare (cm}^2\text{)}}$$

Herbicides were applied 25 days after planting. The experimental layout therefore gave 4 replicate pots of each combination of herbicide type / herbicide concentration / watering regime. Nitrogenase activity of plants in the preliminary experiment was highly variable. For this reason plants were transferred to a growth room (22^{o}C, 17 hour photoperiod) for 5 days before harvest in an attempt to standardize nitrogenase activity of the plants. Plants were harvested 39 days after planting due to the extra 5 days in the growth room. Plants were stored damp in a cold room after acetylene reduction had been carried out until all growth parameters had been measured.

8.3.4.3. Parameters.

Measurements were recorded of number of plants per pot, number of nodules per pot, fresh weight of shoots, fresh weight of roots, total dry weight of plants per pot, and nitrogenase activity by the acetylene reduction assay (see Appendix 5 for method).

Chapter 9.0. Results of *In Vitro* Testing of Herbicide Toxicity on *Rhizobium trifolii* RS102.

9.1. Preamble.

In order to determine if *Rhizobium trifolii* is directly affected by the herbicides under test, *in vitro* experiments were carried out. Several methods of *in vitro* pesticide toxicity testing were used in an attempt to avoid interpretive errors due to inherent bias of any one method. Many workers testing pesticide toxicity toward microorganisms have carried out tests on solid media. Four methods of applying herbicides to *R.trifolii* on solid media were tested, in order to make the results of this study comparable to others already described. Comparison of results of the different methods could be made in order to identify the degree of variation between the methods.

In testing herbicide toxicity *in vitro*, it was attempted to apply herbicides at concentrations approximating those likely to be received by the soil surface under normal agricultural spray regimes. As it is difficult (if not impossible) to calculate levels of herbicide actually reaching the soil after foliar application to a crop, 1x and 10x concentrations were used based on those recommended by the manufacturer. It was assumed that these would represent the maximum levels that could reach the soil surface under the recommended spraying regime.

Experiments involving herbicides incorporated into or spread onto the agar surface were uncountable by 5 days after inoculation due to the mucoid nature of *Rhizobium trifolii*. These experiments were therefore abandoned. Results from well and disc experiments were analyzed, and results are shown in graph 1.0 and 2.0.

9.2. Controls.

Water and acetone control treatments caused no inhibition of rhizobial growth at any time throughout the experiment.

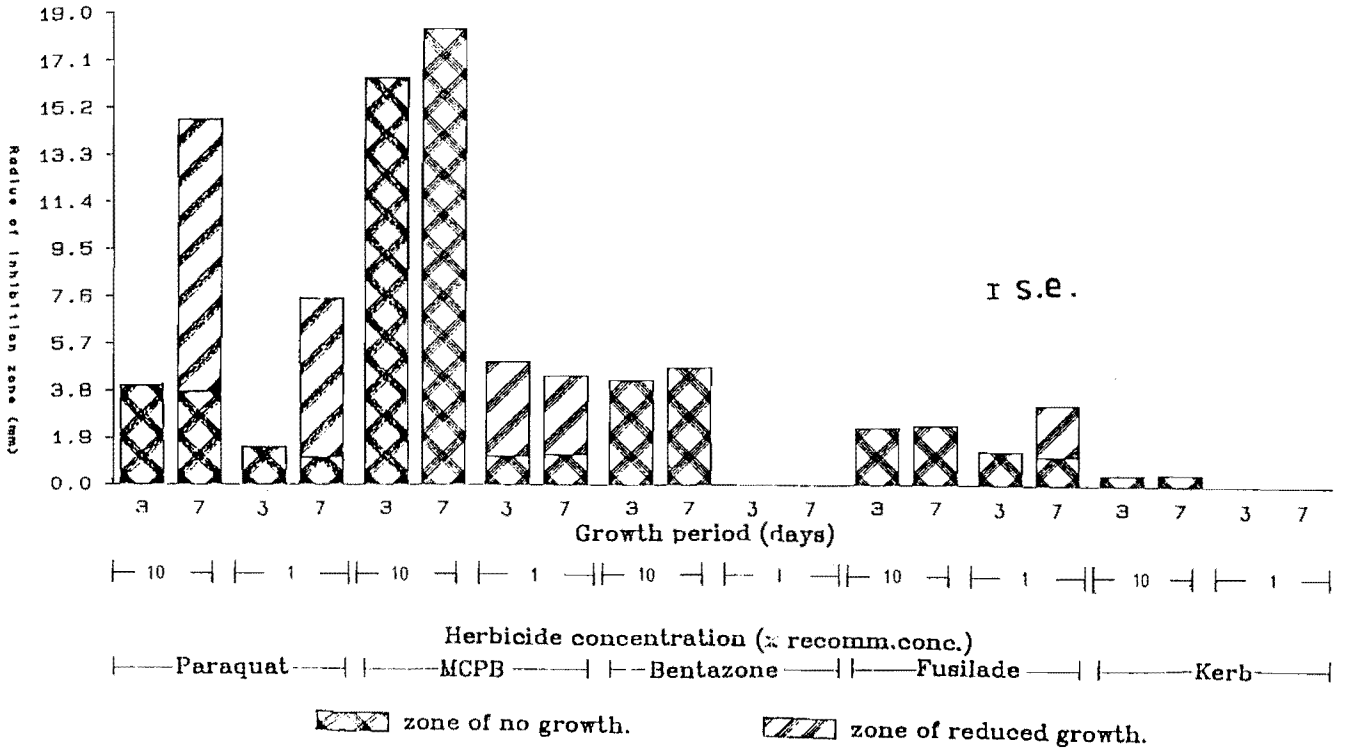
9.3. Toxicity testing of herbicides on *R.trifolii* RS102 on solid medium.

9.3.1. Paraquat.

A clear zone, the result of no rhizobial growth, surrounded wells filled with 10 X concentration of paraquat (graph 1.0). This area of growth inhibition did not alter in size over the 7 days of observation. A translucent zone, interpreted as being a zone of reduced growth further from the well did increase in radius over time. Paraquat applied at 1x concentration in wells created a small (1-2mm radius) clear inhibition zone which also remained constant in size for 7 days (graph 1.0). Paraquat application at 10 X concentration in well experiments affected rhizobial growth to a much greater degree than 1 X concentration of this herbicide applied by the same method (graph 1.0). However inhibition of rhizobial growth caused by paraquat applied on paper discs was similar in degree at both concentrations of paraquat applied (graph 2.0). Larger translucent zones were present around discs containing paraquat than around wells. Paraquat at 1 X concentration gave greater inhibition zones when applied

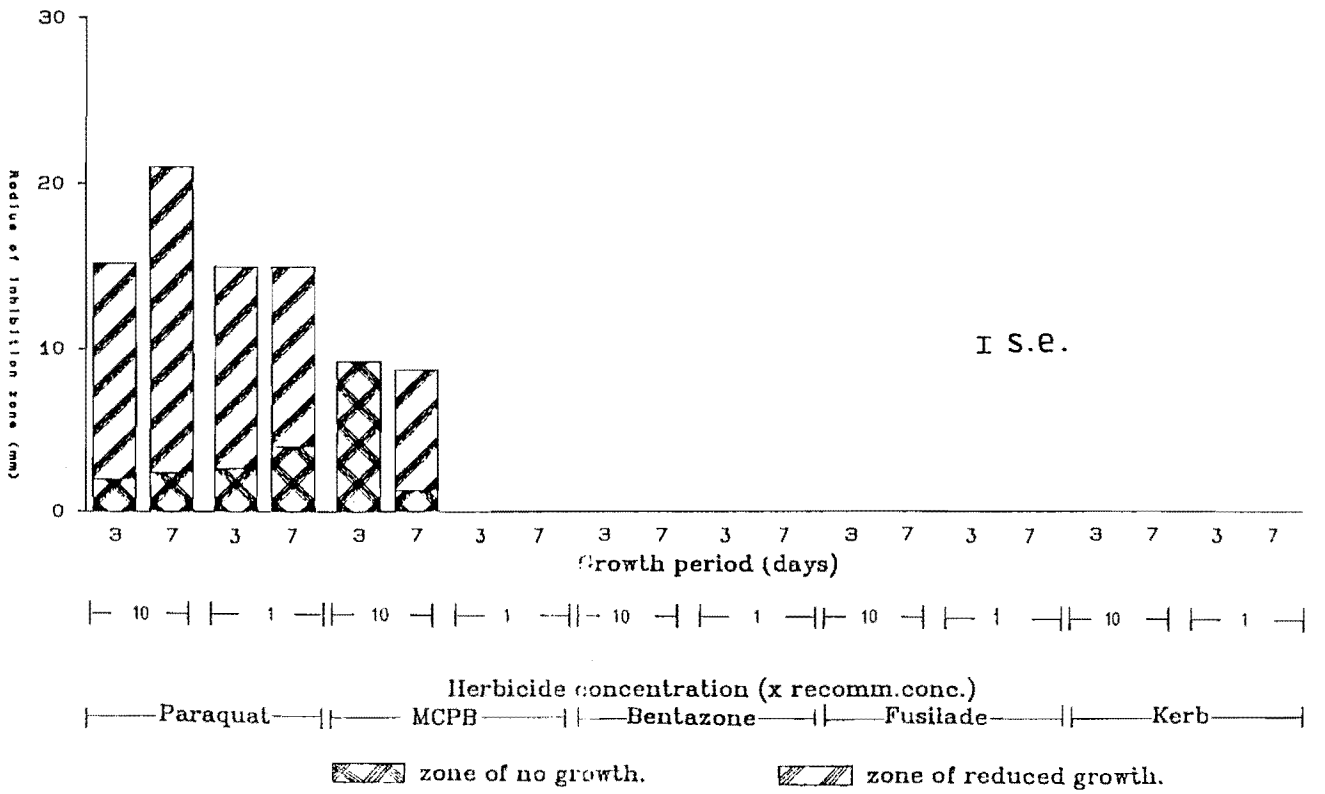
1.0.Inhibition of Growth of R.Trifolii by Herbicide Treatment.

Application of herbicides on wells.



2.0.Inhibition of Growth of R.Trifolii by Herbicide Treatment.

Application of herbicides on discs.



on discs than those resulting from this concentration of paraquat applied in wells (graphs 1.0 and 2.0).

9.3.2. MCPB.

Application of MCPB in wells gave greater inhibition zones than application of this herbicide on paper discs (graph 1.0). MCPB at 1 x concentration in wells caused small clear inhibition zones between 1 & 2mm in radius, and much larger translucent zones (graph 1.0). 1 X concentration of MCPB gave no inhibition when applied on paper discs. MCPB at 10 x concentration significantly inhibited rhizobial growth when applied in wells or on discs. 10 X concentration of MCPB gave much greater inhibition zones than 1 X concentration in both well and disc application methods (graphs 1.0 and 2.0).

Differences in radius of inhibition zones from wells between 3 and 7 days were not significant, indicating the effect of this treatment was bactericidal rather than bacteriostatic (graph 1.0). Clear inhibition zones resulting from disc applications of 10 x concentration of MCPB, became significantly smaller over the 4 day period of observation (graph 2.0). The outer translucent zone encroached on the area previously devoid of growth. This effect implies that the growth inhibition of *Rhizobium trifolii* by MCPB on discs was bacteriostatic, in contrast to the results from wells.

9.3.3. Bentazone.

No inhibition of rhizobial growth was observed as a response to discs treated with bentazone (graph 2.0). 1 x concentration of bentazone applied in wells had no visible effect on rhizobial growth. 10 x concentration of bentazone formed small (4-5mm) inhibition zones about wells (graph 1.0). This inhibition appeared to be dependent on diffusion of the herbicide through the medium, as only application within wells of bentazone in solution caused any growth inhibition. The growth inhibition observed was significant, and no alteration in its degree occurred over the time of observation indicating the effect was bactericidal.

9.3.4. Fusilade.

Fusilade caused small zones of growth inhibition at both concentrations when applied in wells (graph 1.0). 10 x concentration of fusilade caused zones of no growth, however 1 x concentrations caused rhizobial growth to be reduced rather than halted. Rhizobial growth was not affected by application of fusilade on paper discs (graph 2.0). This response indicates that the toxic effect of fusilade was determined by the degree of diffusion of this herbicide through the media.

Inhibition zones formed by fusilade did not alter in size between 3 and 7 days of growth (graph 1.0), hence the toxic effect of this herbicide was bactericidal.

9.3.5. Kerb.

Well application of kerb at 10 X concentration caused small inhibition zones (less than 1mm in radius) (graph 1.0) about wells. This inhibition zone remained unchanged over the period of measurement, indicating a possible bactericidal effect.

No other application of kerb at either 1 x or 10 x concentration inhibited rhizobial growth (graph 1.0 and 2.0).

9.4. Results of liquid medium testing of herbicide toxicity to *Rhizobium trifolii.*

Growth in broth culture is an alternative method which has been frequently used to assess herbicide toxicity toward microorganisms. Liquid culture methods have the advantage over solid medium in that growth can be more accurately quantified by turbidometric means and viable plate counts. Results are presented in appendix 2 and shown in graph 3.0 and 4.0. It was found that testing in liquid media was more reliably recorded by viable plate counts than by densitometric readings from a Spectronic 20.

Statistical analysis of rhizobial growth was used to determine if variation in growth of the bacteria was due to the presence of herbicides in the growth media. A one-way ANOVA and Duncans Multiple Range test both indicated that variation in growth rates of *Rhizobium trifolii* in flasks treated with herbicides at 1 X or 10 X concentrations was due to chance alone. No toxicity at either level of any herbicide or by acetone was recorded.

9.5. Discussion of herbicide toxicity to *R.trifolii* RS102 *in vitro.*

9.5.1. Preamble.

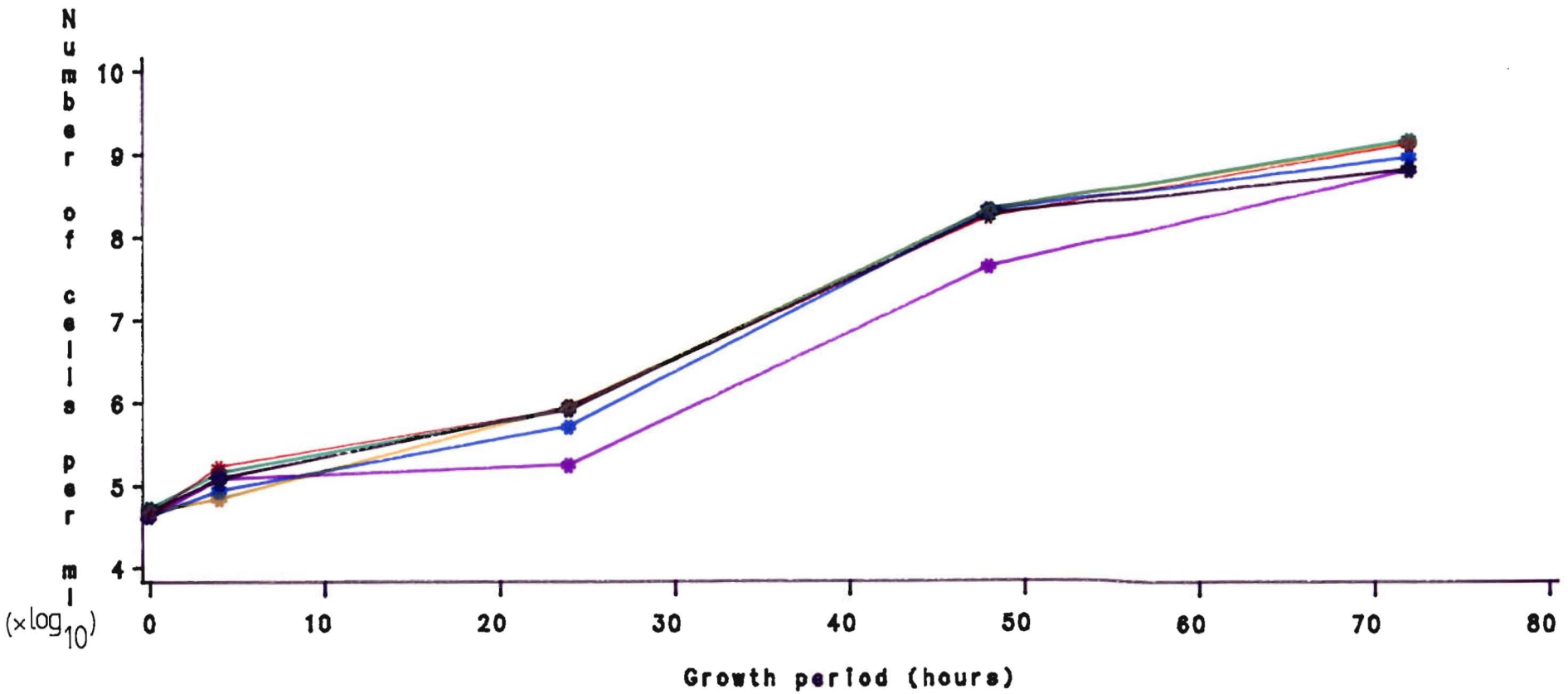
Diatloff (1970), using a slow growing rhizobium, recorded inhibition zones caused by pesticide application at 5 and 10 days for bacteriostatic effects. Brockwell and Robinson (1975) measured inhibition zones around discs of insecticide placed on a lawn of *R.trifolii* after 3 days. Recording growth inhibition of the fast growing *Rhizobium trifolii* at 3 and 7 days after treatment therefore gives a reliable indication of the presence of bacteriostatic and bactericidal toxicity.

9.5.2. Paraquat.

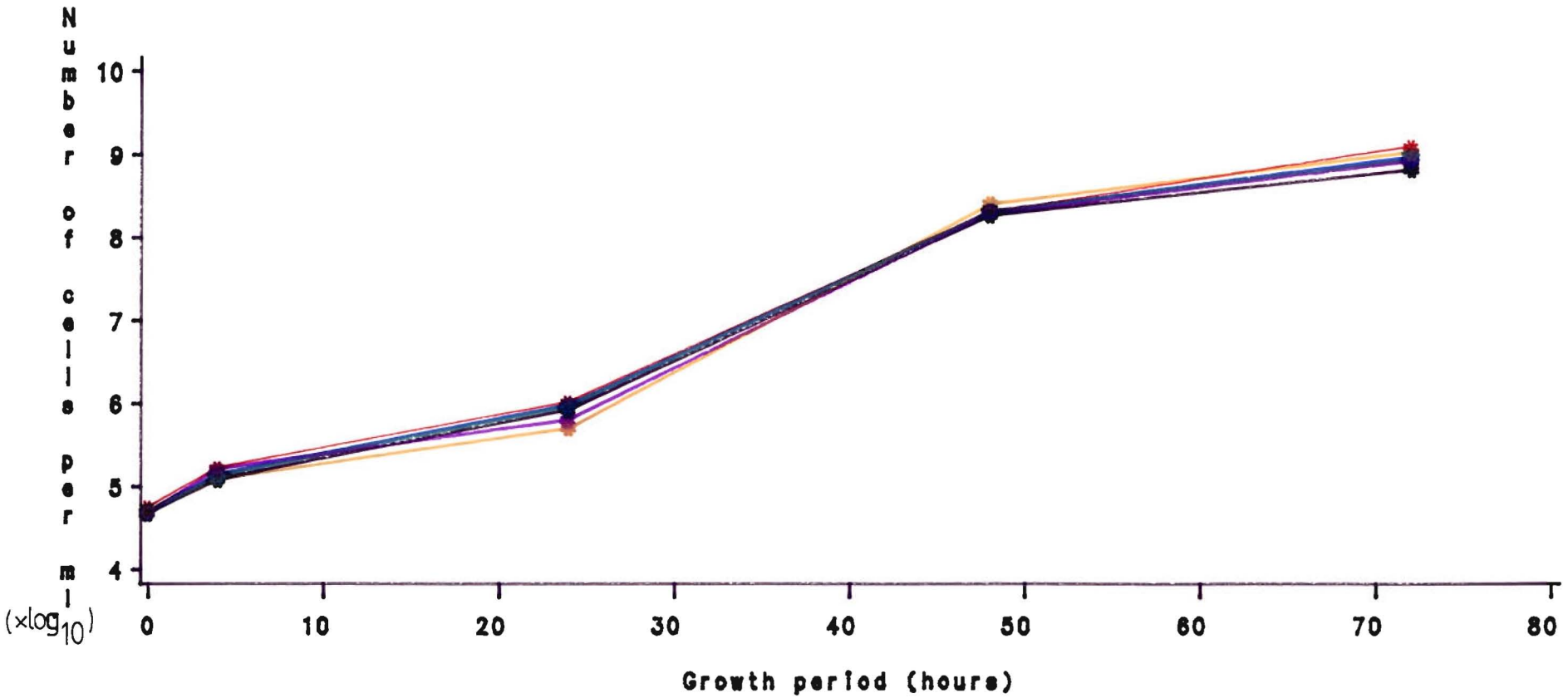
10 x concentration of paraquat gave larger zones of no growth when applied in wells as compared to disc application (graph 1.0 and 2.0). However disc application of paraquat caused zones of reduced growth larger than those formed by well application (graph 2.0). 1 x concentration of paraquat gave greater growth inhibition when applied on discs than when applied in wells (graph 2.0). Both concentrations tested gave similarly sized inhibition zones when applied on discs (graph 2.0), but 10 x concentration resulted in much larger inhibition zones than 1 x concentration when applied in wells (graph 1.0).

Paraquat is known to bind tightly to soil colloids, due to its double positively charged cation. It is possible that binding of the paraquat to agar may prevent dispersion of the herbicide when applied in solution, therefore paraquat applied in wells would not disperse as far as paraquat application on discs, thus explaining the difference in the size of inhibition zones resulting from the two application methods used.

3.0.Growth of R.trifolii in liquid culture.
Herbicides applied at 1x recomm. conc.



4.0.Growth of R.trifolii in liquid culture.
Herbicides applied at 10x recomm. conc.



Diatloff (1970) pointed out that the size of the inhibition zone in culture work may not give a true measure of toxicity to rhizobia since the test depends not only on the sensitivity of the test organism, but also on the concentration and ease of diffusion of the chemical through the agar. The variation in result of the two methods used in this experiment of paraquat toxicity appears to illustrate this point. Diatloff found the size of the zone of inhibition created varied according to the form in which the pesticide was applied. A form with low water solubility gave smaller inhibition zones than one having high water solubility, while application of pesticides in solution gave larger zones of inhibition than dried applications.

Namdeo and Dube (1972) found paraquat above 100ppm doubled the period of lag phase and prolonged the log phase of growth of rhizobia by 24 hours. However this concentration is much greater than the 1 to 3 ppm actually recommended by manufacturers for foliar application of paraquat to clover crops. The respiration of *R.trifolii* was found to be unaffected by paraquat at 10ppm (Grossbard 1970). Manninger *et al.*(1972) treated 20 rhizobial strains of 5 species with paraquat, and found the recommended field levels inhibited growth of 40 % of the strains *in vitro*. This author distinguished 2 types of inhibition of bacterial growth by paraquat. One zone was of weaker growth, the other was of complete growth inhibition, as found in the current experiment. The presence of two types of inhibition zone about the application site of some of the herbicides tested may indicate the position of critical concentrations of herbicide within the agar. The outer radius of the transparent zone may represent the position of the minimum level of herbicide required to reduce rhizobial growth. The boundary between the clear and the translucent zone represents the position of the minimum concentration of herbicide required to halt rhizobial growth.

Brockwell and Robinson (1976) ranked zones of inhibition as clear (=no growth), turbid (=reduced growth) and 0 (=normal growth). An "intensity of inhibition" value was calculated, which equalled the width of the inhibition zone multiplied by the clarity rating. These authors found rhizobial growth on agar was reduced substantially at high concentrations (greater than 10,000ppm) of organo-phosphorous insecticides, whereas nitrogen fixation of clovers grown *in vitro* were unaffected by 1 x and 10 x concentrations of insecticide. It was concluded that the effect of the insecticide was directly on the bacteria, not indirectly via interference with host plant metabolism or the bacterial-plant interaction. However the difference in concentration at which the symbiotic partners were tested determined this outcome. At concentrations equivalent to those at which the plants were tested, the bacteria did not show any growth inhibition.

9.5.3. MCPB.

Rhizobial growth was more inhibited by application of MCPB in wells, than application on discs. 10 x concentration of MCPB applied on discs proved to be bacteriostatic, the clear zone becoming grown over by 7 days (graph 2.0). However

well application of the same level gave an increased zone of no growth by 7 days (graph 1.0). Well application of herbicides were made in solution, whereas paper discs soaked in herbicides were dried prior to placement on the rhizobial lawn. Hence the different result may be due to lack of diffusion of MCPB from discs through the medium, whereas MCPB diffuses to a greater extent from wells. Concentrations of herbicides applied were calculated to approximate levels reached in soil under recommended spraying regimes. Hence, in the case of MCPB, well application probably gives a truer measure of the toxicity as it allowed dispersal throughout the plate. MCPA had no effect on growth of 3 species of rhizobia up to 800ppm *in vitro* (Gillberg 1971), a much higher level than was found toxic by Vintikova *et al.*(1965).

Kaszubiak (1966) found MCPB ('tropotox') inhibited growth of 5 rhizobial strains in doses above 1000ppm. This author suggested that even doses exhibiting no effect on growth of bacteria may influence their metabolism. However as the recommended level of MCPB is less than 10ppm, the concentrations applied in these experiments are much greater than those likely to contact rhizobia in the field.

Fletcher and Raymond (1956), and Fletcher *et al.*(1956) showed that examination of *R.trifolii* after 4 days incubation indicated an adverse affect on growth by 50-100 ppm of MCPB, but when the cultures were allowed to grow for a further 10 days the bacterium recovered and growth was affected only by concentrations of 500 ppm and above. These levels are much greater than those tested in the present experiment. However an inhibition of growth of *R.trifolii* on solid medium when exposed to 5-50ppm MCPB was found, although these concentrations did not affect growth of the same bacteria in liquid culture.

9.5.4. Bentazone.

Bentazone gave small inhibition zones of between 4 and 6 mm in radius when applied at 10 x concentration in wells (graph 1.0). No other treatment of bentazone gave any inhibition of rhizobial growth. It appears unlikely, therefore, that bentazone at 2.5 to 25 ppm would inhibit rhizobial growth *in vivo*. Lindström *et al.*(1985) found the much higher concentration of 100 ppm of bentazone did not affect the growth of *Rhizobium* strains.

9.5.5. Fusilade.

Fusilade caused zones of no growth when applied at 10 x concentration and smaller zones of reduced growth when applied at 1 x concentration in wells (graph 1.0). However disc application of fusilade did not cause any growth inhibition of rhizobia (graph 2.0). Fusilade has a very low solubility in water. As herbicides applied on discs were dried, while well applications of herbicides were in aqueous form, it is probable that little movement of fusilade occurred from disc applications. Therefore well experiments probably represent the field situation better than disc application experiments, as *in vivo* herbicides are in solution. Hence fusilade may have the potential to damage rhizobial growth.

9.5.6. Kerb.

Kerb treatment at 10 x concentration in wells caused inhibition zones of less than 1 mm in radius (graph 1.0), while all other kerb applications did not affect rhizobial growth. It therefore appears improbable from these results that kerb would affect rhizobial growth in soil.

9.5.7. Liquid medium testing.

Testing of herbicide toxicity toward *R.trifolii* in liquid media at the same concentrations showed that none of these herbicides adversely affect growth of the bacteria as recorded by viable plate counts (graphs 3.0 and 4.0). The difference in this result from that of plate experiments is probably due to the herbicide in liquid medium being completely dispersed. Application of herbicides in wells or by paper discs on solid media sets up a concentration gradient, hence inhibition zones under these conditions reflect the response of the bacteria to a much greater concentration of the herbicide than that to which bacteria are exposed in liquid media.

9.5.8. Concluding comments.

The majority of the work done on toxicity of pesticides to symbiotic associations has found the microsymbiont to be unaffected by pesticides at concentrations approximating those applied in the field (Table 1-3).

Grossbard (1970b) exposed rhizobia to herbicides during growth, then used these cultures to inoculate white clover. The treated rhizobia were inoculated onto host plants both before and after being washed free of excess herbicide. Unwashed inocula resulted in a decrease in nodulation and in some cases severe damage to the plants. Washed inocula did not exhibit any affect against plant growth or nodulation. Hence the pesticide activity was primarily against the plant, and not the rhizobia. It is possible that even doses exhibiting no effect on growth of the bacteria may influence their metabolism. Fletcher and Raymond (1956) found sub-bacteriostatic doses of phenoxyherbicides reduced the ability of *R.trifolii* to form symbioses with clover.

From the results (graphs 1.0 to 4.0), the herbicides tested may be ranked in order of toxicity as paraquat being more toxic than MCPB, followed by fusilade and bentazone, and kerb as the least toxic.

The concentrations of herbicide to which rhizobia were exposed in the present experiment are probably much higher than those contacting rhizobia in the soil. Interactions of herbicides with soil colloids, leaching, chemical and microbial decomposition and plant uptake all lower pesticide levels in soil below that applied. Hence this experiment indicates that paraquat and MCPB have the potential to affect rhizobial growth in soil, although it is unlikely that bentazone, fusilade or kerb would act directly on rhizobial growth.

Chapter 10.0. Results and Discussion of *In Vitro* Study of Herbicide Toxicity to *Trifolium repens* and its Symbiosis with *Rhizobium trifolii* RS102.

10.1. Preamble.

The symbiosis between *Trifolium repens* and *Rhizobium trifolii* is a very complex and delicate relationship. The presence of toxic chemicals such as herbicides in the growth environment of the plant may affect either member of the symbiosis, or the relationship itself. In order to identify the target of herbicide toxicity within this relationship, *in vitro* methods (ie. plants grown on agar under aseptic conditions) were employed to test herbicide activity against the symbiotic partners both individually and together.

Each combination of application time / herbicide type / herbicide concentration / nitrogen presence or absence / rhizobial presence or absence were replicated 6 times. This arrangement resulted in four possible combinations of plants grown on supplied nitrogen and inoculated with rhizobium, ie, $^{+}N^{+}R$, $^{+}N^{-}R$, $^{-}N^{+}R$ and $^{-}N^{-}R$. When results appeared variable, the experiment was repeated.

The number of plants required for this *in vitro* experiment were too great to be harvested at any one time, therefore the experiment was set up as two trials. The first trial consisted of toxicity testing of herbicides when plants were treated 3 days after germination, while the second trial contained all plants treated with herbicides 21 days after germination. Each trial was staggered, with each group treated with a specific herbicide being harvested 4 days apart. In this way variation between the trials and groups could be detected by analysis of control values.

Results of this *in vitro* experiment are tabulated in Appendix 3 and presented in graphs 1.1 to 5.7. Only the most statistically significant are considered here. Unless stated otherwise, significance is $p< 0.05$.

10.2. Controls.

10.2.1.Water controls.

Analysis by Pearsons Correlation Coefficients showed no relationship existed between initial radicle length and plantlet weight, and final fresh and dry weight of untreated plants at the time of harvest in this experiment. Therefore it can be assumed that the sample used was random, and growth of plants was not related to genetic predisposition.

10.2.1.1. Effect of application time.

Variation in growth due to the herbicide treatment group or application time trial in which the plants were grown was determined by Analysis of Variation (ANOVA) of control parameters.

Control plants of the trial treated with herbicides at 3 weeks had significantly higher values of fresh weight ($p=.001$) (graph 1.2), dry weight ($p<.001$) (graph 1.1) and lateral root numbers ($p<.001$) than those control plants of the trial treated at 3 days but leaf numbers were significantly greater ($p<.005$) for plants in the trial grown for treatment at 3 days. Variation between time trials is probably due to

fluctuation in growth room temperature and light intensity over the time of the experiment. It was later found that considerable fluctuation of environmental variables occurred within the growth chamber used for this experiment. ANOVA's of herbicide treatment experiments compare herbicide effects to the group's own controls, hence this variation does not impair the analysis.

Shoot height, root length, nodule number (of 1 to 2 per plant,graph 4.2) and acetylene reduction (graph 3.3) were not significantly different between the untreated controls of the two time trials (3 and 21 day treatments). A preliminary study of growth of white clover plants under these conditions showed the growth of control plants within the experiment to be of a normal level.

10.2.1.2. Effect of nitrogen.

The presence of an available nitrogen source in the growth medium significantly decreased nodule numbers (graph 2.4) and nitrogenase activity as shown by the C_2H_2 reduction assay, as has been reported by other authors (Carroll and Gresshoff 1983). All parameters of plant growth (weight and extension) were stimulated by nitrogen (graph 2.5 & 5.4).

10.2.1.2.a. Effect of nitrogen and application time.

Plant fresh weight ($p<.001$), dry weight ($p<.001$), lateral root numbers ($p=.001$) and leaf numbers ($p<.001$) were stimulated by the presence of nitrogen in the media significantly more in the untreated control plants grown for treatment at 3 weeks than those for treatment at 3 days (graph 1.3). This effect also may be attributed to the variation in growth room environmental variables during the experiment. There was no significant difference between the two time trials in these parameters among plants lacking supplied nitrogen (graph 1.3).

10.2.1.3. Effect of rhizobial inoculation.

Inoculation of plants with *R.trifolii* significantly increased shoot height ($p<.001$), root length ($p<.001$) (graph 1.5), fresh weight (graph 1.4), dry weight (graph 3.7), leaf numbers ($p=.001$), and shoot fresh weight. Only plants inoculated with *R.trifolii* RS102 had nodules and exhibited nitrogenase activity.

10.2.1.3.a. Effect of rhizobial inoculation and nitrogen.

Plants provided with nitrogen and inoculated with rhizobia (ie.$^{+}N^{+}R$) did not have greater shoot height than plants provided with nitrogen alone (ie.$^{+}N^{-}R$). However rhizobial inoculation significantly ($p<.01$) stimulated shoot height of plants lacking an alternative nitrogen source. This result indicates that stem and petiole extension is primarily affected by nitrogen in the growth medium. Rhizobial inoculation only acts on this parameter under those conditions when an alternative nitrogen source is unavailable.

10.2.2. Acetone Controls.

Trials in which acetone was used as a solvent for the herbicide application, contained controls treated with concentrations of acetone equivalent to those received by herbicide treated plants. These controls ensured that measurement

was made of the possible effects of the solvents used on plant growth. Acetone controls were statistically compared to water controls to detect any effect of this solvent on white clover development. ANOVA showed acetone significantly lowered the number of lateral roots of plants when compared to water controls ($p<.001$). No other parameter was affected by acetone at the levels applied. It is reasonable to assume that all other significant effects are due to herbicidal activity.

The relevant results of growth of control plants will be stated at the beginning of each section, in order to highlight comparisons to herbicide treated plants.

10.3..The effect of paraquat on white clover *in vitro*.

10.3.1. Effect of paraquat concentration.

Both 1 x and 10 x concentrations of paraquat significantly inhibited all parameters measured at both application times (graphs 1.1 to 1.6).

10.3.2. Effect of application time.

Analysis of untreated plants showed that those grown in the trial for treatment 3 weeks after germination had significantly greater values of fresh weight, dry weight and lateral root numbers than those treated 3 days after germination. However the control plants of the 3 day trial had significantly more leaves (see section 10.2.1.1). All growth parameters of plants treated with paraquat were significantly less when treated 3 days after germination than when treated 3 weeks after germination (graph 1.1,1.2,1.3 & 1.5). Nodulation was completely inhibited by paraquat applied 3 days after germination.

10.3.2.a.Effect of paraquat concentration and application time.

10 x concentration of paraquat applied 3 days after seed germination inhibited shoot height, root length, fresh weight (graph 1.2), dry weight (graph 1.1), lateral root numbers and leaf numbers to a greater extent than 1 x concentration of paraquat. Application of 10 times concentration of paraquat 3 weeks after germination significantly affected shoot fresh weight ($p<.001$), but not root fresh weight of white clover plants. Application of 1 x concentration of paraquat 3 weeks after germination did not exert as severe an effect as did 10x concentration (graph 1.1 and 1.2).

10.3.3. Effect of nitrogen.

Plant growth was significantly stimulated in control plants by the presence of nitrogen, while nodulation and nitrogenase activity was suppressed. The presence of nitrogen in the media did not significantly inhibit nodulation of paraquat treated plants, as paraquat treatment in most cases completely inhibited nodulation of both ^{+}N and ^{-}N plants.

10.3.3.1. Effect of nitrogen and application time.

Plants treated with paraquat 3 days after germination were not stimulated by nitrogen in the growth media (graph 1.3). Shoot height, root length, fresh weight, dry weight, lateral root and leaf numbers of paraquat treated +N plants were not significantly greater than paraquat treated -N plants (graph 1.3), however nitrogen did stimulate growth of plants treated with paraquat 3 weeks after germination.

10.3.3.2. Effect of nitrogen and paraquat concentration.

Paraquat at 10 x concentration inhibited growth stimulation by nitrogen to a greater extent than at 1 x concentration (graph 1.3).

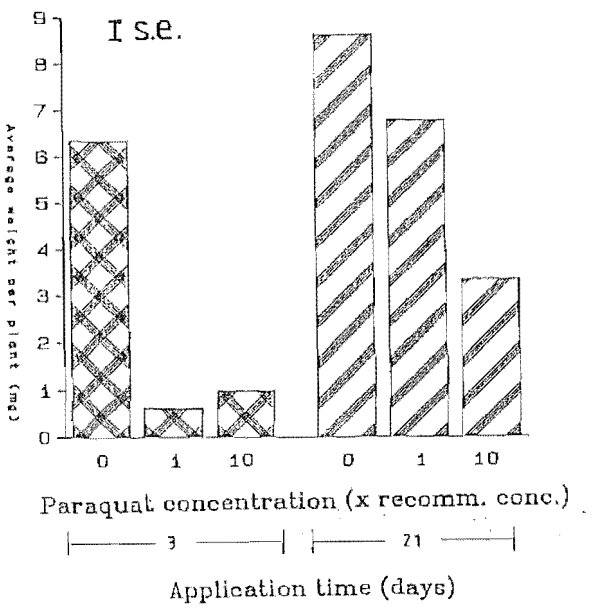
10.3.4. Effect of rhizobial inoculation.

Rhizobial inoculation normally increases shoot height,

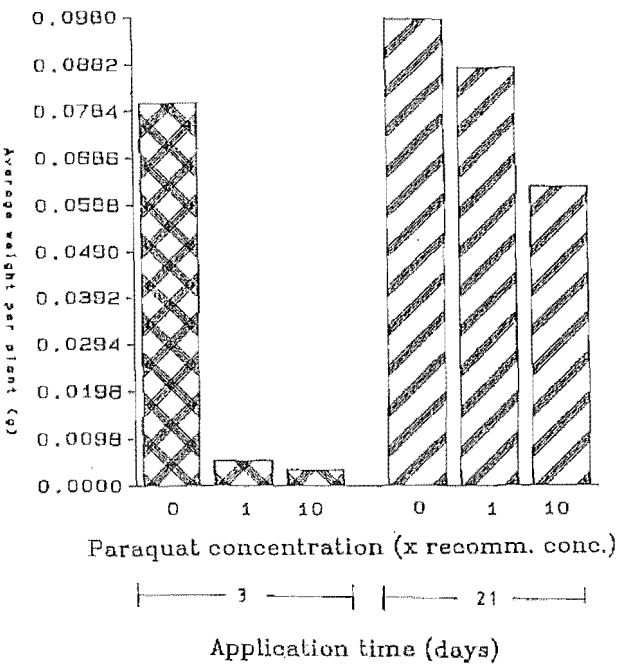
root length, fresh weight, dry weight and leaf number. Plants treated with paraquat overall did not show significantly greater fresh weight when inoculated with rhizobia as compared to plants grown without rhizobia (graph 1.4).

Paraquat

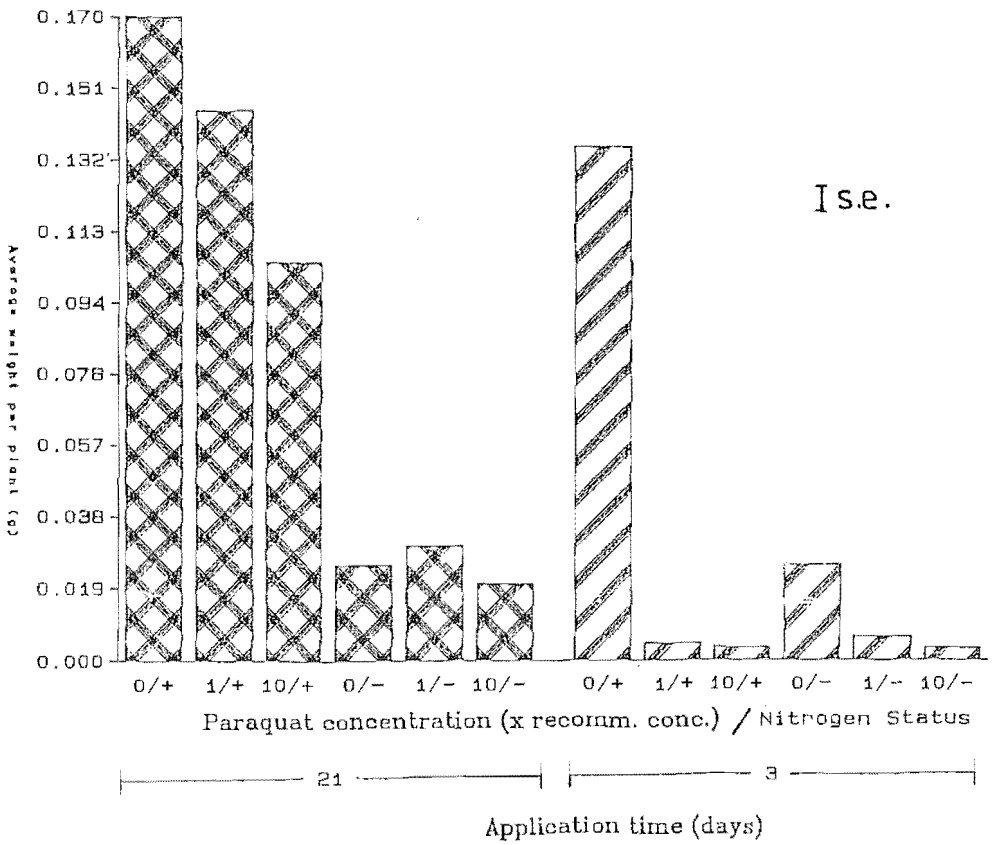
1.1. Effect on Dry Weight.



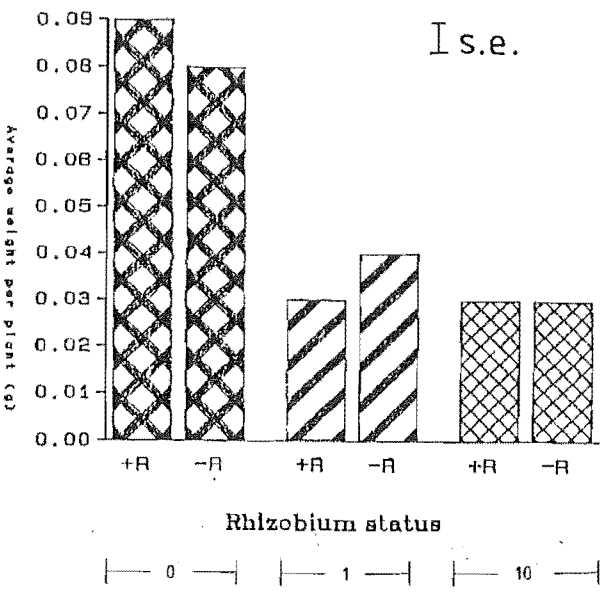
1.2. Effect on Fresh Weight.



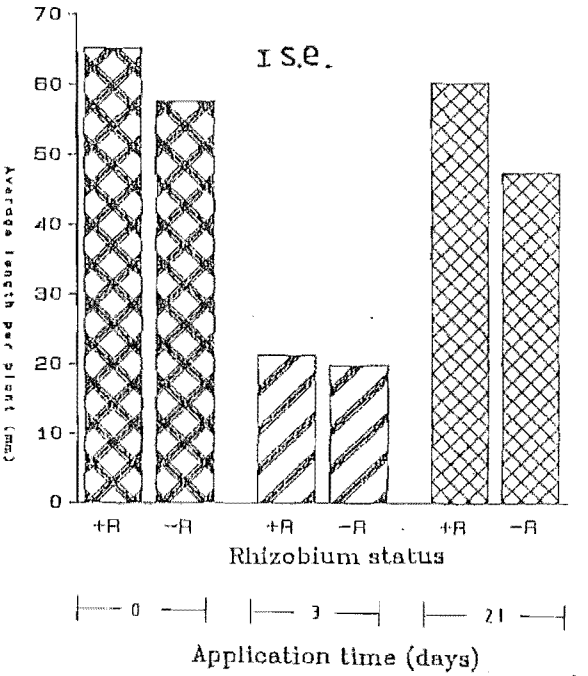
1.3.Influence of Nitrogen on Fresh Weight.



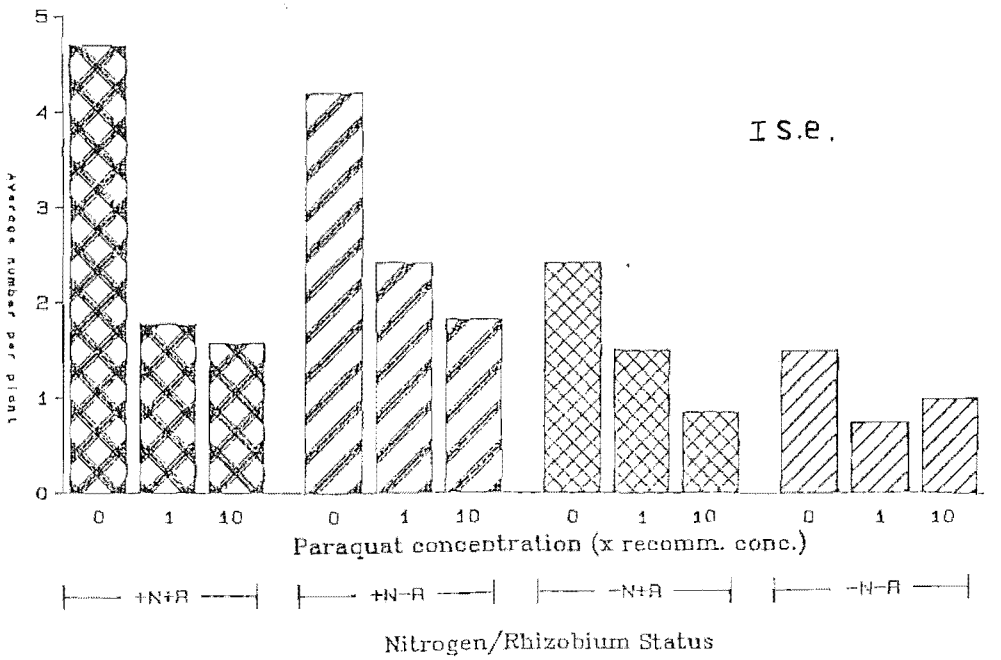
1.4.Influence of Rhizobium on Fresh Weight.



1.5.Influence of Rhizobium on Root Length.



1.6.Influence of Nitrogen and Rhizobium on Leaf Number.



10.3.4.1. Effect of rhizobial inoculation and application time.

Rhizobial inoculated plants treated with paraquat at the seedling stage did not have greater root length than plants treated at the same time and lacking rhizobial inoculation (graph 1.5). Paraquat treatment 3 weeks after germination did not have as severe an effect, rhizobial inoculation showing a significant stimulatory effect on plant growth parameters.

10.3.4.2. Effect of rhizobial inoculation and nitrogen.

Rhizobial inoculation of untreated plants had no stimulatory effect on shoot height when plants were supplied with nitrogen in the growth media. Following paraquat application plants supplied with both nitrogen and rhizobia (ie.$^{+}N^{+}R$) not only lack increased values of shoot height but leaf numbers are also no greater in these plants than in plants with nitrogen alone (ie.$^{+}N^{-}R$) (graph 1.6).

10.3.5. Discussion of the effects of paraquat on white clover *in vitro*.

Paraquat is a rapid contact herbicide causing white clover plants to show rapid loss of chlorophyll following treatment. Rapid loss of shoot weight of plants treated 3 weeks after germination reflects paraquat's dessicant activity. Treatment with paraquat at the seedling stage damaged white clover to the same degree regardless of the concentration applied. However at later stages of growth higher levels of paraquat exerted a greater effect on plant growth and plant response to nitrogen than recommended concentrations. Plants treated with paraquat 3 days after germination were severely inhibited (graph 1.1,1.2,1.3,1.5). Treatment of plants 21 days after germination did not cause as severe a response, although these plants also showed significant growth inhibition.

Plants treated with paraquat at the seedling stage did not show any response to the presence of nitrogen in the medium (graph 1.3), as paraquat treatment at this stage appears to inhibit the plants ability to utilize nitrogen. Plants treated with paraquat 3 weeks after germination did show stimulation in growth in response to nitrogen (graph 1.3), however this response was less than that of control plants, probably being mainly a reflection of plant growth prior to the herbicide treatment. Paraquat treatment at this time also appears to inhibit plant metabolism or uptake of nitrogen.

Application of paraquat inhibited rhizobial stimulation of shoot fresh weight. Paraquat acts as a dessicant, hence loss of fresh weight of plant shoots following treatment with paraquat is possibly due in part to water loss rather than loss of dry matter, therefore inoculated plants treated with paraquat 21 days after germination did not give the increased fresh weight that normally occurs. Plants treated at the seedling stage did not develop nodules, hence rhizobia could not stimulate plant growth through providing fixed nitrogen.

Paraquat inhibits white clover development very rapidly. Paraquat acts by competing for electron flow from the primary electron acceptor of Photosystem I. The herbicide is converted to the free radical by reduction, subsequent autoxidation yielding

the original ion. The free radical itself does not appear to be the primary toxicant. During autoxidation of the free radical to the ion, hydrogen peroxide (H_2O_2), superoxide radical (O_2^-), hydroxyl radical (OH^-) and singlet oxygen are formed. The hydroxyl radical is believed to be responsible for paraquat induced lipid peroxidation, membrane damage and phytotoxic symptoms (Dodge 1971).

As paraquat has such a severe and rapid effect on plant photosynthesis, effects of paraquat on nodulation and nitrogenase activity are thought to be due to loss of photosynthetic metabolites essential for nodule functioning. However the data of this experiment tends to indicate a direct effect of paraquat on white clover nodules. Nodule numbers and nitrogenase activity are severely reduced by paraquat, and therefore inoculation with rhizobia does not stimulate plant growth. Only 1 x concentration of paraquat, 21 days after germination did not completely inhibit nodulation of white clover in this experiment.

10.4. Effect of MCPB on white clover *in vitro*.

10.4.1. Effect of MCPB application.

1 x concentration of MCPB significantly inhibited root length ($p<.001$) and dry weight ($p=.016$) of white clover plants(graph 2.1). MCPB treatment at 10 x concentration also inhibited root length ($P<.001$) (graph 2.2) and dry weight ($p<.001$) (graph 2.1) as well as shoot height ($p<.001$), fresh weight ($p<.001$) (graph 2.3), lateral root number and leaf numbers ($p=.001$) of white clover *in vitro*. Nodulation and acetylene reduction were not significantly affected by either concentration of MCPB.

10.4.2. Effect of application time.

Untreated plants of the 3 week trial had significantly greater fresh weight, dry weight and lateral root numbers than the control plants of the 3 day trial. However control plants of the 3 day trial had significantly more leaves per plant. Plants treated with MCPB 3 days after germination also had significantly lower average shoot height and root length than those treated 3 weeks after germination (graph 2.2). Leaf numbers of plants treated with MCPB 3 days after germination were also lower than normal.

10.4.2.1. Effect of MCPB concentration and application time.

Both concentrations of MCPB applied to white clover at the seedling stage were toxic to shoot height, root length (graph 2.2), fresh weight (graph 2.3) and dry weight (graph 2.1) of white clover plants. However MCPB at 10x concentration applied 21 days after germination was significantly more harmful ($p<.05$) to white clover than recommended levels. 10 x concentration of MCPB was also significantly more harmful to leaf numbers when applied 3 days after germination then when applied 21 days after germination.

10.4.3. Effect of nitrogen.

Nitrogen supplied in the growth medium stimulated all growth parameters of untreated plants. Growth of MCPB treated plants did not differ from controls in their response to nitrogen. However nodules of plants treated with 1x concentration of MCPB were not significantly suppressed by nitrogen (graph 2.4). Nodule counts of plants grown on medium supplemented with nitrogen may be unreliable due to the difficulty in nodule identification caused by root deformation.

10.4.3.1. Effect of nitrogen and application time.

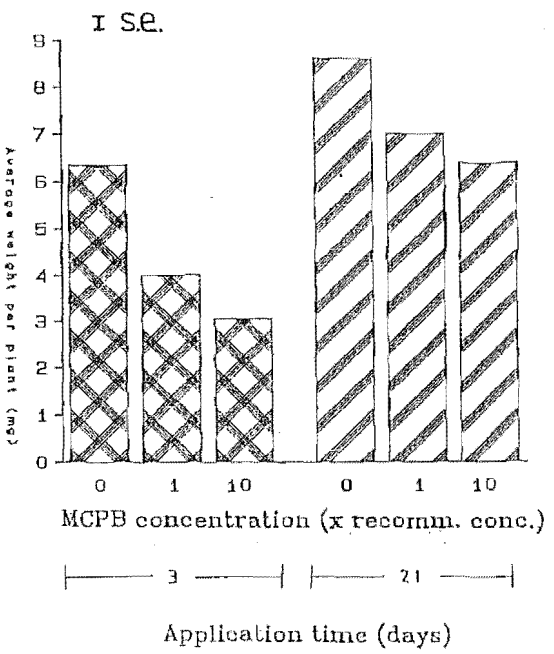
Within controls, supplied nitrogen showed a greater stimulatory effect on weights and root and leaf number of plants of 3 week trials than on untreated plants of 3 day trials. Plants treated with MCPB 3 days after germination were not stimulated by nitrogen as much as control plants (graph 2.5).

10.4.3.2. Effect of nitrogen and MCPB concentration.

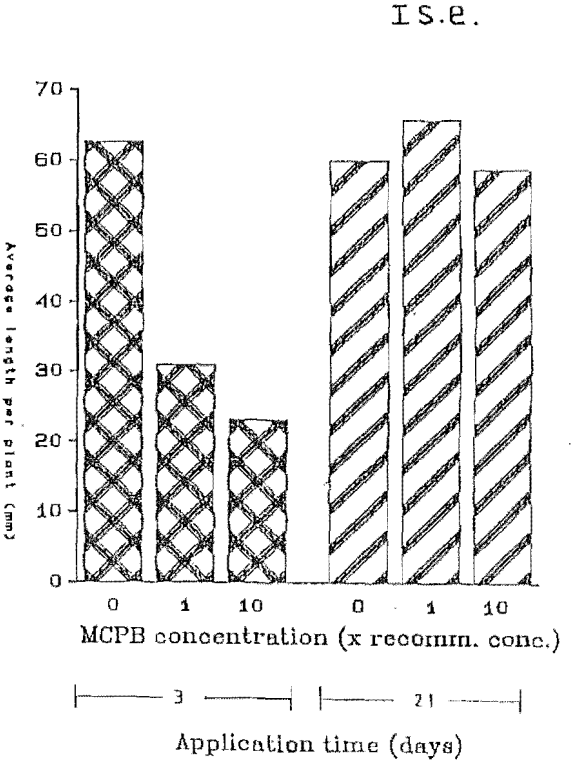
Plants grown on medium containing nitrogen showed greater inhibition of shoot height, lateral root and leaf number by MCPB than plants grown without supplied nitrogen. For example, MCPB at 1 x concentration lowered the average shoot height of plants grown on medium containing nitrogen by 80 %, whereas shoot height of white

MCPB

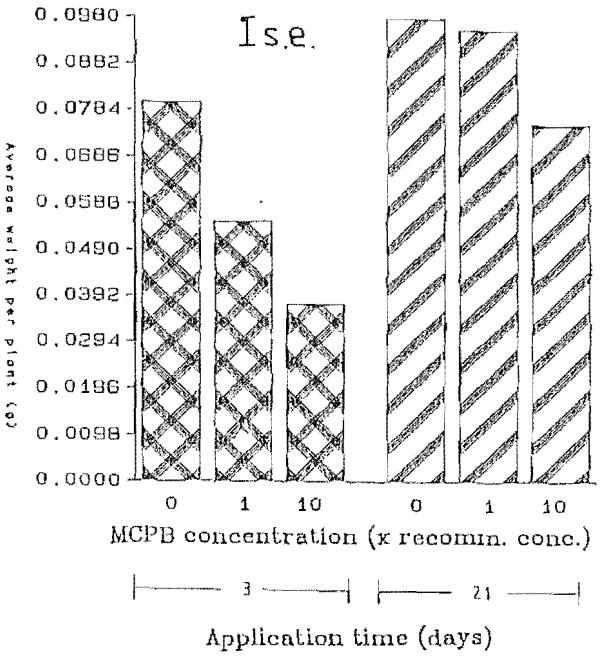
2.1. Effect on Dry Weight.



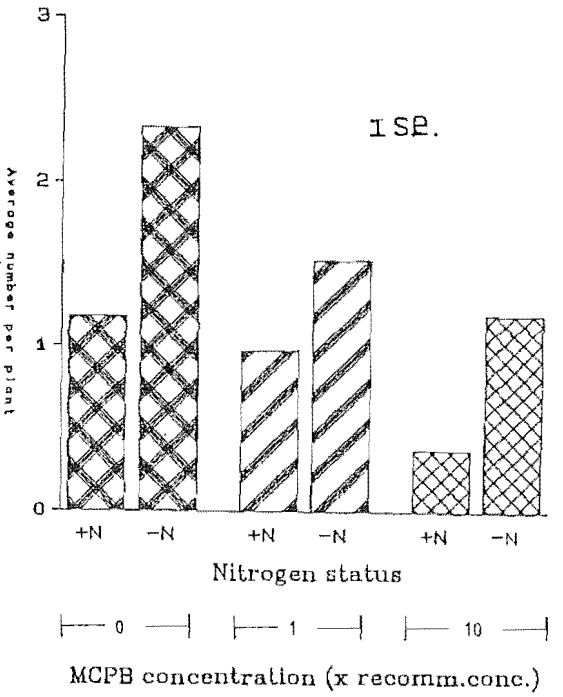
2.2. Effect on Root length.



2.3. Effect on Fresh Weight.

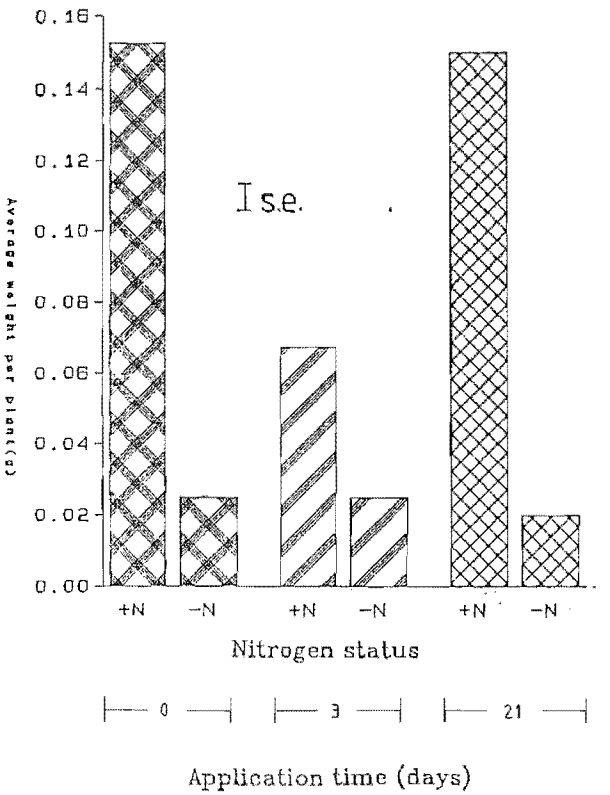


2.4.Influence of Nitrogen on Nodulation

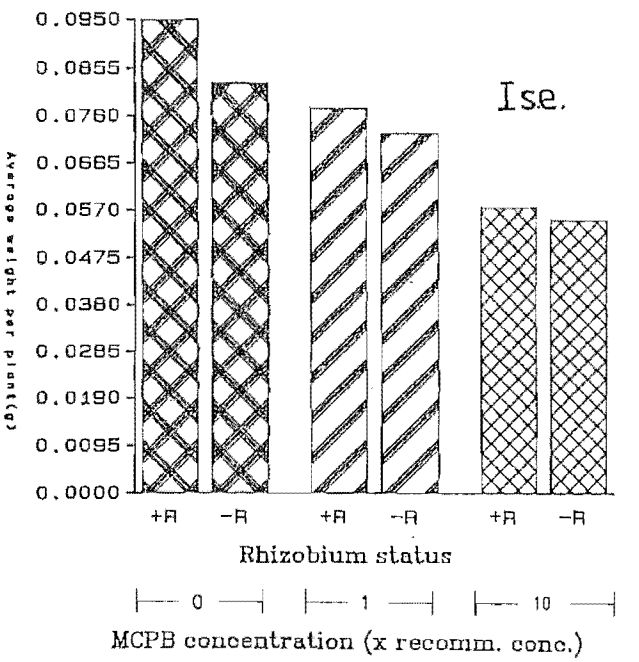


MCPB

2.5.Influence of Nitrogen on Fresh Weight



2.6.Influence of Rhizobium on Fresh Weight.



Colour Plate 1. Four week-old white clover (*Trifolium repens*) grown *in vitro* and treated with MCPB. Note severe root stunting. +N = Nitrogen was supplied in the growth medium. +R = Plants were inoculated with *Rhizobium trifolii* RS102.

clover grown on medium lacking nitrogen was not affected by this concentration of MCPB.

10.4.4. Effect of rhizobial inoculation.

Untreated plants had greater shoot height, root length, fresh weight, dry weight, leaf numbers and shoot fresh weight when inoculated with rhizobia. However shoot height, root length, fresh weight, dry weight and leaf numbers of MCPB treated plants did not show a significant stimulation in response to rhizobial inoculation (graph 2.6).

10.4.4.1. Effect of rhizobial inoculation and MCPB concentration.

Rhizobial inoculation significantly increased lateral root number ($p=.048$) and shoot fresh weight ($p=.03$) of plants treated with 1 x concentration of MCPB. However inoculated plants treated with 10 x concentration of MCPB had less lateral roots than controls, whereas plants lacking rhizobial inoculation had similar numbers of lateral roots as untreated uninoculated plants. MCPB application at 10 x concentration inhibited the growth stimulation of white clover that normally occurs after inoculation with *R.trifolii* (graph 2.6).

10.4.4.2. Effect of rhizobial inoculation and nitrogen.

Inoculation of untreated plants with rhizobium stimulates shoot height only when the plant is not supplemented with nitrogen. When plants were treated with MCPB, rhizobial inoculation only stimulated lateral root numbers when nitrogen was not present in the growth medium.

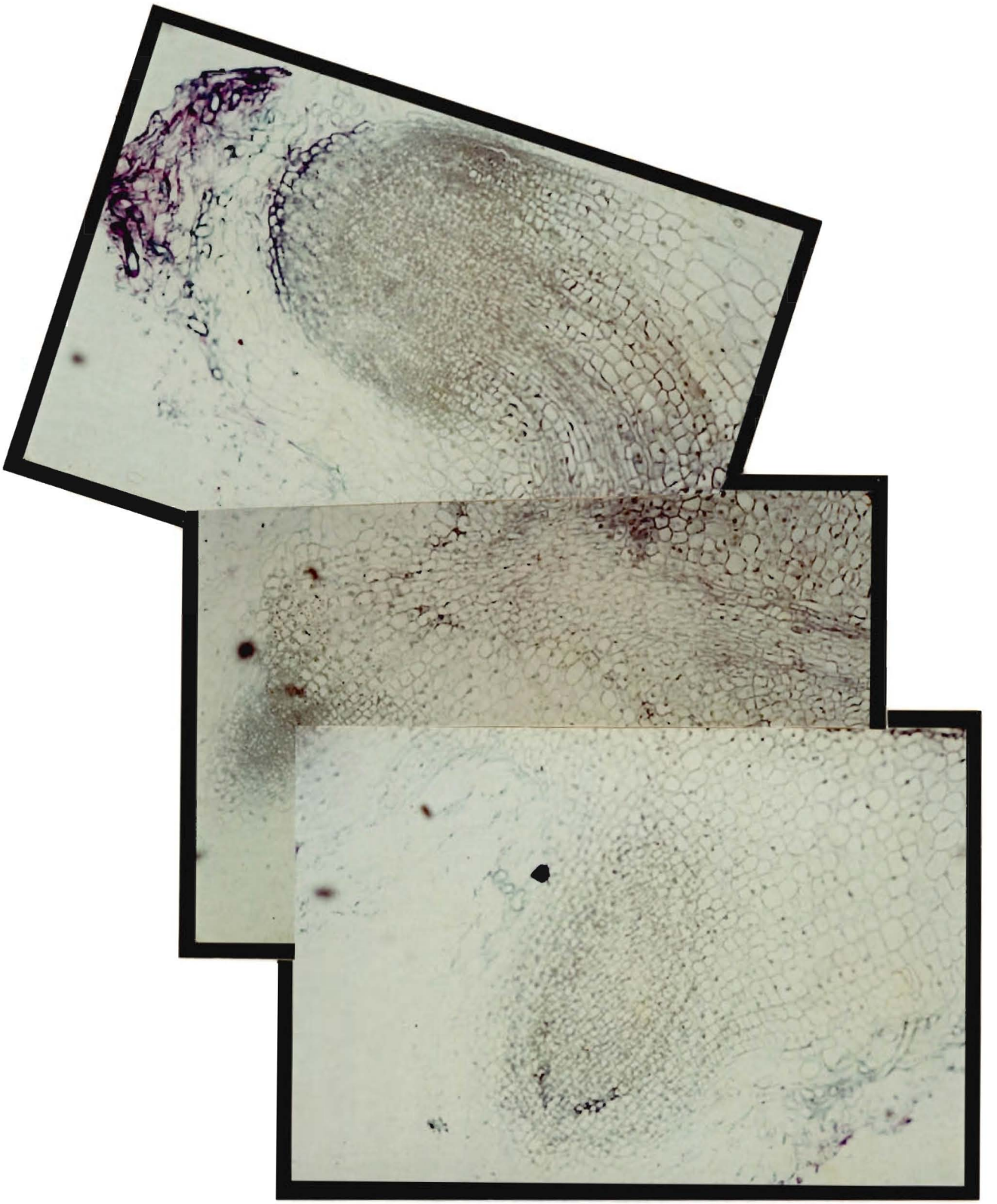
10.4.5. Discussion of the effects of MCPB on white clover *in vitro*.

The response to MCPB reported here is in many ways identical to that reported for MCPA - the active form of MCPB (Gorter & Zweep 1964). The selectivity of MCPB is based on the presence in plants of a B-oxidation pathway. Plants possessing this pathway will convert MCPB to the toxic form MCPA. MCPA causes marked deformation of white clover roots but no epinasty of the petioles nor abnormalities of the leaflets, while MCPB does not affect white clover (Fletcher *et al.*1956). White clover showed a response to MCPB similar to that described for MCPA (color plate 1). Fletcher et al. noted that rate of movement of MCPA into aerial parts was reduced by conditions favoring a low rate of transpiration. In the present experiment, as in that of Fletcher et al. the humidity was high, indicated by the presence of droplets of water on the sides of the petri dish, hence the transpiration rate was low. Consequently there may be little movement of the herbicide in the plant.

MCPB was particularly effective in stunting root growth (color plate 1) although total plant dry weight was also inhibited by both concentrations tested. This inhibition of dry weight without a concomitant decrease in fresh weight indicates that MCPB may halt nutrient uptake but increase water uptake of plants. MCPA is known to cause roots to lose the ability to take up nutrients (Gorter & Zweep 1964).

Application of MCPB at different times indicated that white clover plants were a great deal more sensitive to the herbicide when applied at early stages of

Colour Plate 2. Light micrograph montage of a longitudinal section of a stunted lateral root apex from a four week-old white clover (*Trifolium repens*) plant treated with MCPB. Tissue is stained with toluidine blue. Note large areas of cell division with little cell elongation. x 350.



development (graph 2.1,2.2 and 2.3). Plants grown without nitrogen or rhizobial inoculation did not exhibit any root deformity. MCPB also inhibited rhizobial stimulation of lateral root development to a greater degree in plants supplemented with nitrogen than those lacking an available nitrogen supply. Hence MCPB activity appears to be limited to actively growing plants, indicating a meristematic site of activity as has been reported (Audus 1964).

Nodule numbers and nitrogenase activity were not found to be significantly inhibited by MCPB treatment. However both these parameters were much lower in MCPB treated plants than their respective controls (graph 2.4). It is probable that MCPB does interfere with nodulation and nitrogenase activity to the extent that these factors no longer stimulate plant growth, as rhizobial inoculation did not significantly increase growth of MCPB treated plants (graph 2.6).

An intriguing response of white clover to MCPB treatment at 1 x concentration was the lack of significant inhibition of nodulation by the presence of nitrogen, although nodulation of plants grown on N supplemented medium was less than that of plants grown on medium lacking nitrogen (graph 2.4). This is possibly due to the known affect of MCPA mentioned above of causing roots to lose the ability to take up nutrients. Hence nitrogen may have no effect as plants can no longer absorb it from the medium. This response is also shown in the lack of stimulation of plant growth by nitrogen when plants were treated with 10 x concentration of MCPB.

The effect of MCPA is known to be greater on plants of higher nutritional status (Gorter & Zweep 1964). Growth of plants treated with MCPB 3 days after germination was inhibited when grown on medium supplemented with nitrogen. Root deformation was also more severe in plants grown with nitrogen (color plate 1). As MCPB interferes with uptake of minerals by roots, lack of uptake of nitrogen by plants grown on nitrogen supplemented media causes a greater inhibition of plant growth relative to control plants than the identical effect on plants lacking nitrogen supplementation.

Growth inhibition by MCPB appears to be primarily through inhibition of root function, as root tissue was visibly distorted, and nitrogen supplementation failed to stimulate plant growth or suppress nodulation of MCPB treated plants. Hence plants do not receive required levels of nutrients due to root activity being inhibited. Ljunggren and Martensson (1980) found MCPA in root medium at 1/2 and 1 times recommended concentrations caused alterations of the entire development of the root system of *Trifolium pratense*, a result very similar to that found following MCPA treatment of white clover by Fletcher *et al.*(1956) and after MCPB treatment in the present experiment (color plate 2).

10.5. The effect of bentazone on white clover *in vitro.*

10.5.1. Effect of bentazone application.

Overall bentazone applied at the two different concentrations produced no significantly different effect for any parameter (graph 3.1).

10.5.2. Effect of application time.

Untreated plants grown for the trial treated 21 days after germination had significantly more fresh weight, dry weight and lateral root numbers than those of the trial treated 3 days after germination. However control plants of the 3 day trial had significantly more leaves per plant than those of the 3 week trial.

Following bentazone application 3 days after germination,lateral root numbers of white clover plants were found to be significantly greater than controls at harvest 4 weeks after germination. Plants treated with bentazone at the seedling stage had greater root length ($p<.01$) and nitrogenase activity ($p<.01$) than plants treated at 3 weeks old (graph 3.3). Root fresh weight of plants treated 3 weeks after germination was also significantly ($p=.044$) increased by bentazone at 10x concentration.

10.5.2.1. Effect of bentazone concentration and application time.

Bentazone significantly increased root length ($p=.002$) and acetylene reduction ($p=.002$) when applied to white clover plants at the seedling stage.(graph 3.3). 1 x concentration of bentazone significantly increased fresh weight (graph 3.1), dry weight (graph 3.2) and lateral root numbers of plants treated 3 days after germination, while 10 x concentration of bentazone significantly increased plant fresh weight when applied 3 weeks after germination.

10.5.3. Effect of nitrogen.

10.5.3.1. Effect of nitrogen and application time.

Nitrogen did not stimulate shoot height, root length or fresh weight of plants treated with bentazone 3 days after germination to as great an extent as untreated plants (graph 3.4). However bentazone treatment 21 days after germination did not suppress nitrogen stimulation of these parameters.

10.5.3.2. Effect of nitrogen and bentazone concentration.

Bentazone treatment of plants grown with supplied nitrogen significantly increased nodulation at the 1 x concentration, whereas 10 x concentration of bentazone stimulated nodulation of plants grown on medium lacking nitrogen ($p=.039$) (graph 3.5).

10.5.4. Effect of rhizobial inoculation.

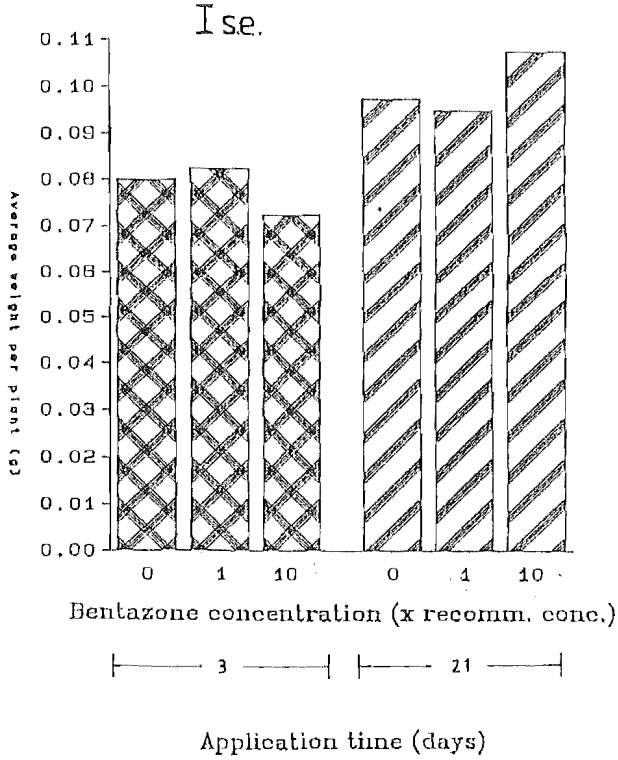
Inoculation of untreated plants with rhizobia increased shoot height, root length, fresh weight, dry weight, leaf numbers, nodulation and shoot fresh weight. Bentazone altered the effect of rhizobial inoculation on root length and dry weight. Plants inoculated with rhizobia did not have greater root length or dry weight than plants lacking rhizobial inoculation (graph 3.6).

10.5.4.1. Effect of rhizobial inoculation and application time.

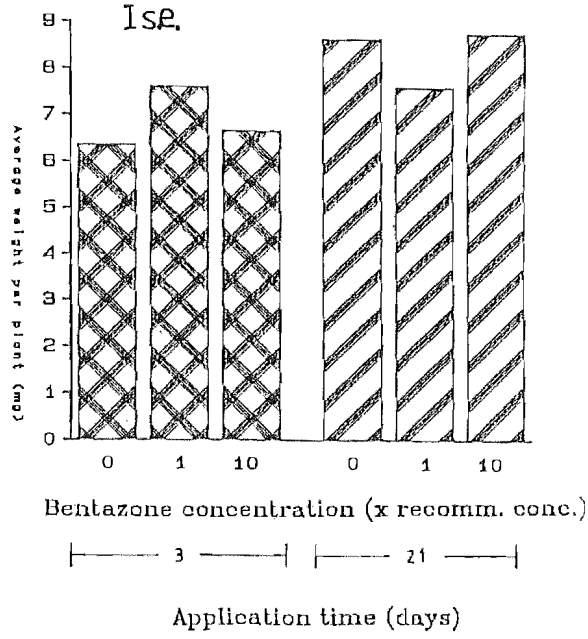
Plants treated with bentazone at 21 days, showed a significant increase in dry weight ($p=.01$) when inoculated with rhizobia. However rhizobial inoculation did

Bentazone

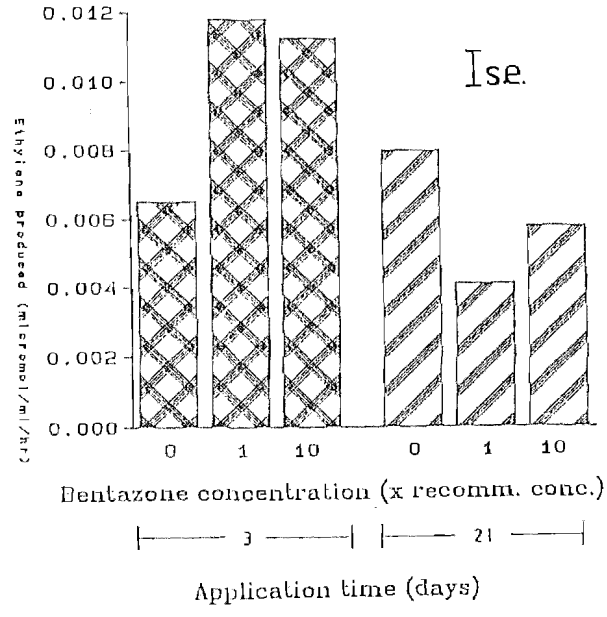
3.1. Effect on Fresh Weight.



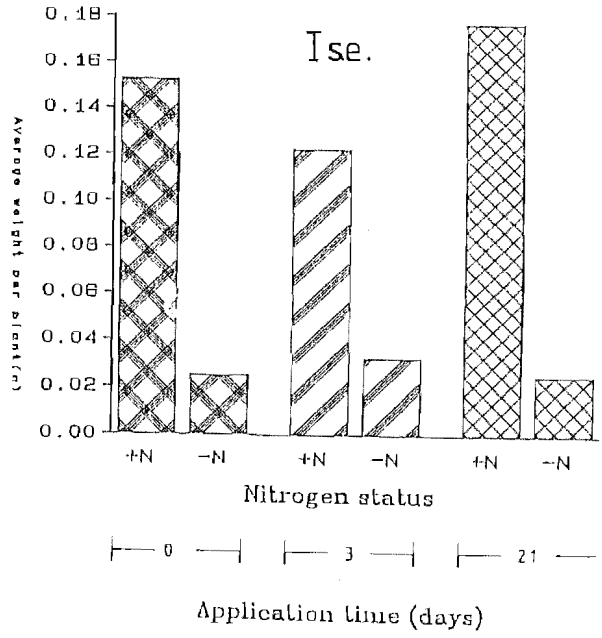
3.2. Effect on Dry Weight.



3.3. Effect on Nitrogenase Activity

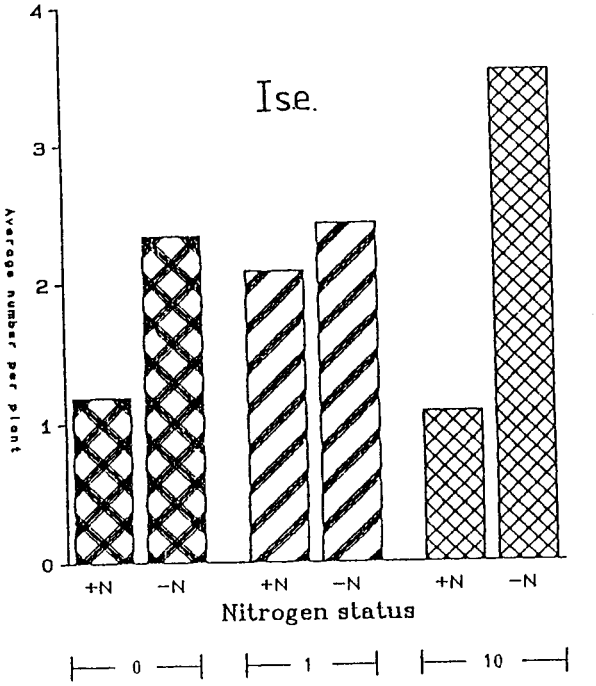


3.4.Influence of Nitrogen on Fresh Wei

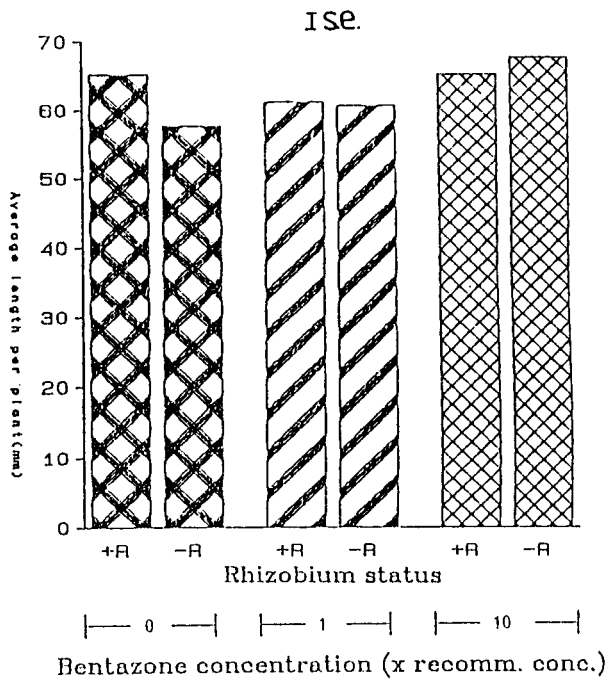


Bentazone

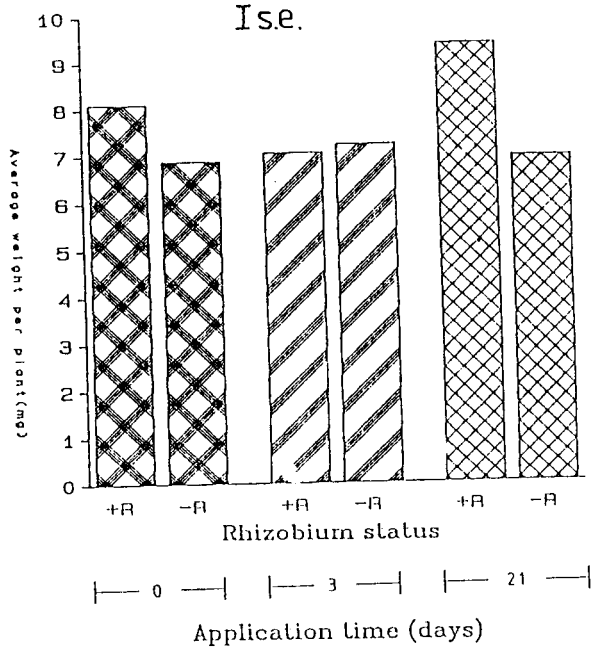
3.5.Influence of Nitrogen on Nodulation.



3.6.Influence of Rhizobium on Root Length.



3.7.Influence of Rhizobium on Dry Weight.



not increase dry weight of plants treated with bentazone at the seedling stage (graph 3.7). Root length of plants treated with 1 x concentration of bentazone 21 days after germination was stimulated by rhizobial inoculation. However all other treatments of bentazone inhibited root length response to *R.trifolii.*

10.5.4.2. Effect of rhizobial inoculation and nitrogen.

Rhizobial inoculation did not stimulate root length or leaf numbers of bentazone treated plants supplied with nitrogen. Bentazone appears to interfere with rhizobial stimulation of plant growth when nitrogen is available in a free form.

10.5.5.Discussion of the effect of bentazone on white clover *in vitro.*

Bentazone is a double ringed structure containing 2 nitrogen atoms per molecule. It is not persistent in soil, 5ppm applied to a loamy sand was found to completely break down within 15 weeks (BASF Technicial Communication). Application of 1 x concentration of bentazone 3 days after germination stimulated plant growth to a greater extent than 3 week application, however 10 x concentration of bentazone did not stimulate plant growth as much as 1 x concentration. Plants treated with bentazone 3 days after germination and grown without nitrogen in the medium were not stimulated to as great an extent by inoculation with rhizobia as untreated plants. Bentazone treatment appears to interfere with rhizobial mediated stimulation of root length, leaf numbers and dry weight of white clover plants. Hence a high concentration may exert some toxicity against white clover.

Bentazone did not completely inhibit nitrogen stimulation of plant growth, although nitrogen stimulation was less in plants treated 3 days after germination (graph 3.4). However bentazone did inhibit rhizobial stimulation of plant growth (graph 3.6), particularly when plants were treated with the herbicide during the seedling stage. Rhizobial inoculation did stimulate dry weight of plants treated with bentazone 3 weeks after germination (graph 3.7). This effect is possibly due to stimulation of growth by rhizobial inoculation that occurred prior to bentazone application. Harvest of some plants at the time of herbicide application would allow determination of the degree of growth before and after treatment.

Nodulation of plants lacking an available nitrogen source was stimulated by 10 x concentration of bentazone. Also lack of rhizobial inoculation did not inhibit root length of bentazone treated plants, as occurred in controls. The presence of small amounts of nitrogen at early stages of growth of nodulated plants is known to stimulate nodule formation at later stages (Richardson *et al* 1957). Bentazone stimulated white clover growth particularly when applied at early stages of growth and is therefore similar in many ways to the activity of nitrogen. These results indicate that breakdown of bentazone may be releasing enough nitrogen to stimulate plant growth and inhibit rhizobial activity.

10.6. The effect of fusilade on white clover *in vitro.*

10.6.1. Effect of application time.

Untreated plants of the 3 weeks treatment trial had significantly greater fresh weight (graph 4.1), dry weight and lateral root numbers than the plants of the 3 day trial, however untreated plants of the 3 day trial had significantly more leaves per plant. Plants treated with fusilade 3 days after germination had significantly lower levels of root length and nitrogenase activity (graph 4.3) than plants treated 3 weeks after germination. Also, plants treated with fusilade 3 days after germination did not have significantly more leaves than those treated at 3 weeks, as occurred in controls.

10.6.1.1. Effect of fusilade concentration and application time.

Root length, fresh weight ($p<.001$) (graph 4.1), dry weight ($p<.001$) (graph 4.4) and lateral root numbers ($p<.001$) were increased by 1x concentration of fusilade 3 days after germination. All other combinations of application time and fusilade concentration inhibited plant growth.

10.6.2. Effect of nitrogen.

Nitrogen in the growth medium of plants treated with fusilade showed the normal effects of this nutrient in stimulating plant growth parameters and suppressing nodulation. However nitrogen application did not significantly suppresses acetylene reduction of plants exposed to fusilade.

10.6.2.1. Effect of nitrogen and fusilade concentration.

Plants grown on media supplemented with nitrogen were more affected in shoot height, fresh weight (graph 4.6), dry weight and lateral root numbers by 1x concentration of fusilade than plants lacking available nitrogen. Plants lacking an available nitrogen source did not show as large a difference between control and fusilade treated plants for these parameters.

10.6.3. Effect of rhizobial inoculation.

Control plants have significantly higher values of shoot height, root length, fresh weight, dry weight, leaf numbers and shoot fresh weight when inoculated with rhizobia. Plants treated with fusilade did not respond to rhizobial inoculation with increased fresh weight (graph 4.7), dry weight and shoot fresh weight, due to inhibition by fusilade of nitrogenase activity (graph 4.3).

10.6.3.1. Effect of rhizobial inoculation and application time.

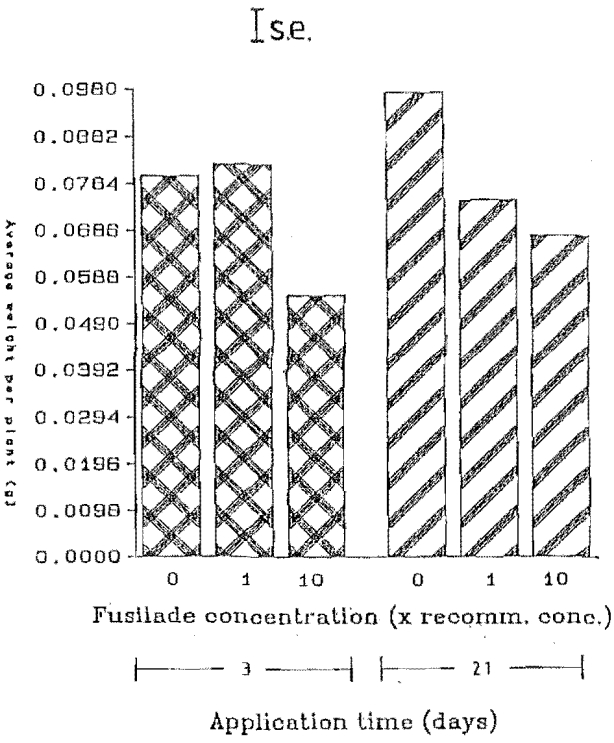
Plants treated with fusilade 3 days after germination did not have greater leaf numbers when inoculated with rhizobia. This effect is probably due to the inhibition of nitrogenase activity by fusilade, hence rhizobial inoculation does not provide the nitrogen to aerial plant parts normally fixed by active nodules.

10.6.4. Discussion of the effect of fusilade on white clover *in vitro.*

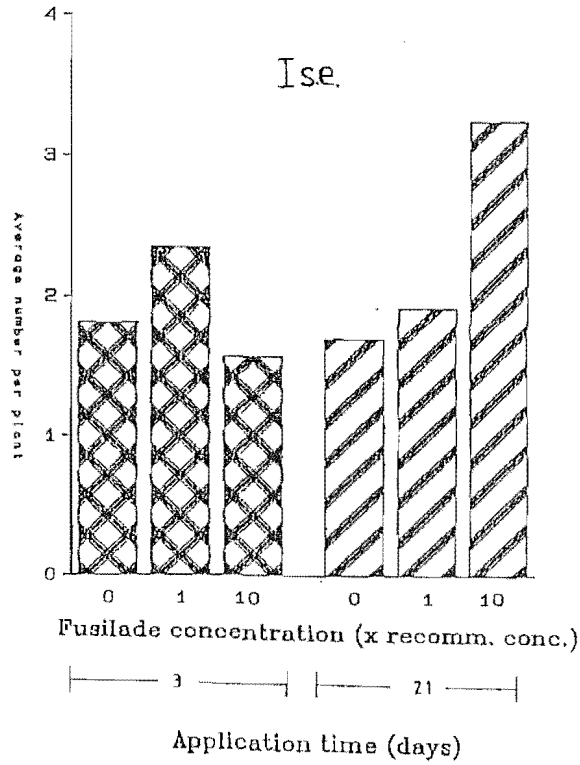
Fusilade has been reported to adversely interfere with ATP production (Plowman *et al.*1980). Fusilade inhibited growth when applied at high concentrations or shortly before harvest, however 1 x concentration of fusilade applied at the seedling stage stimulated plant weight. Fusilade contains one nitrogen atom in each molecule of

Fusilade

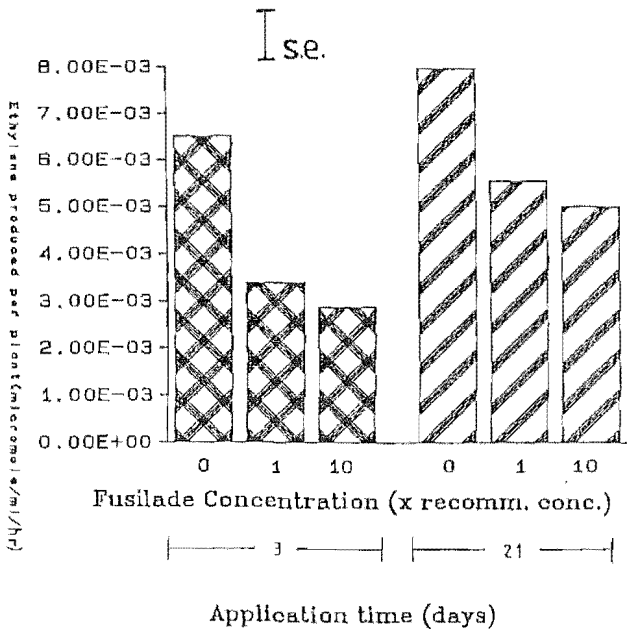
4.1. Effect on Fresh Weight.



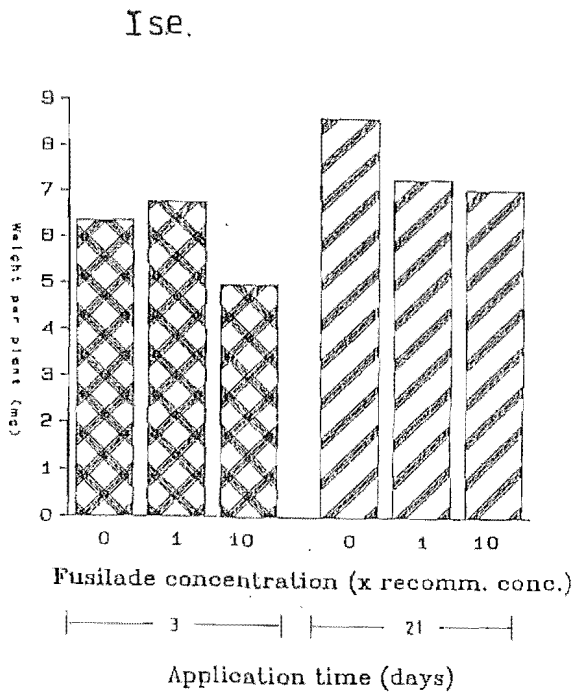
4.2. Effect on Nodulation.



4.3.Effect on Nitrogenase Activity.

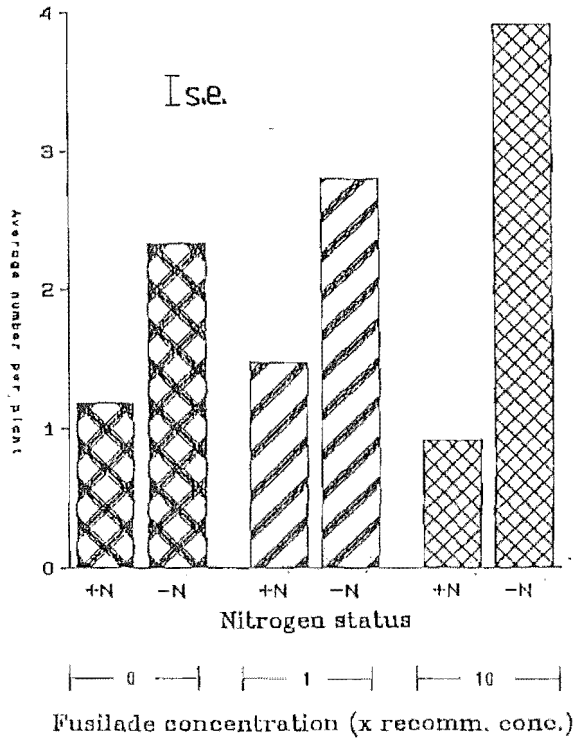


4.4. Effect on Dry Weight.

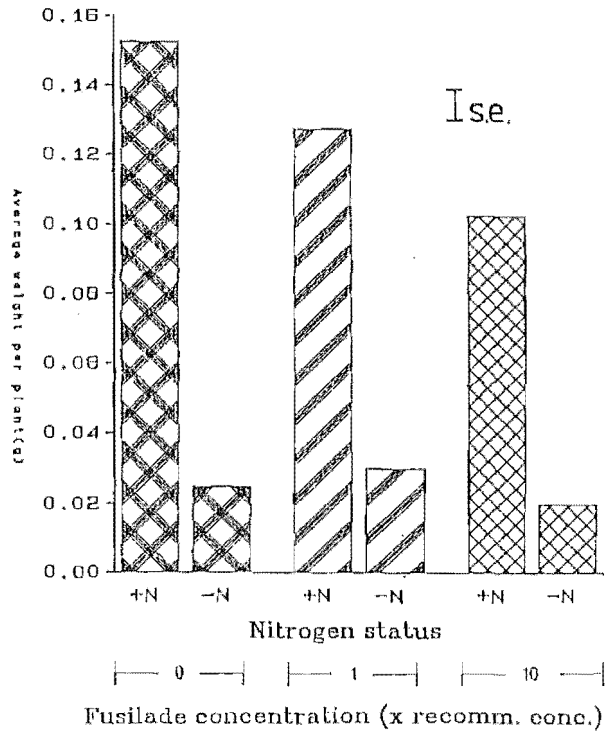


Fusilade

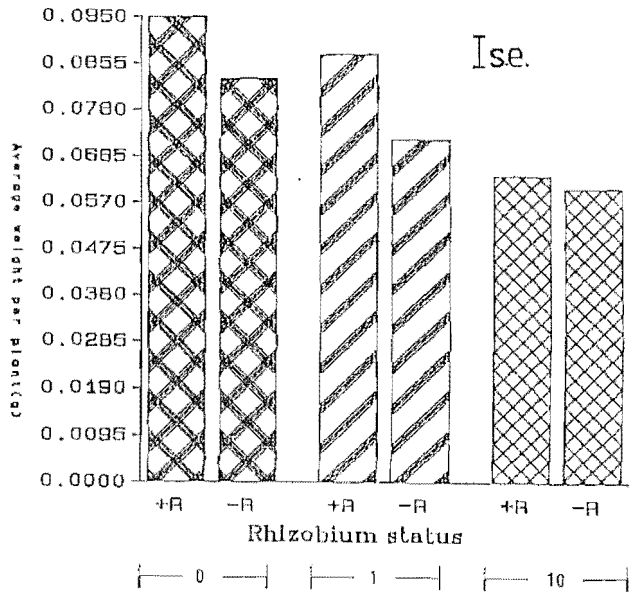
4.5.Influence of Nitrogen on Nodulation



4.6.Influence of Nitrogen on Fresh Weight.



4.7.Influence of Rhizobium on Fresh Weight.



herbicide and is reported to have a 1/2 life in soil of 3-12 weeks (Plowman *et al.*1980). It is possible that at low concentrations fusilade may chemically degenerate, releasing enough nitrogen to cause a slight stimulation of plant growth. Fusilade's toxic effect may, therefore, be short lived, with plants overcoming immediate slight growth inhibitions.

Fusilade prevented nitrogen inhibition of nitrogenase activity. As growth of the rhizobial partner was not affected by this herbicide *in vitro*, this result suggests it is the plant partner that determines nitrogen regulation of nodule activity. Fusilade also exerted a strong inhibition of nitrogenase activity although nodulation was unaffected. Nitrogenase requires ATP for activity, therefore interference of fusilade in ATP synthesis would be expected to exert an inhibitory effect on this enzyme, which is possibly responsible for the lack of stimulation of growth by rhizobial inoculation, as rhizobial inoculation would normally stimulate plant growth via nitrogen fixation. This lack of affect on nodulation (graph 4.2) indicates that fusilade does not damage rhizobial infectivity or clover plants potential to be nodulated.

Vigorously growing plants (those with nitrogen supplied in the medium) were more inhibited by fusilade than plants lacking nitrogen supplementation. Hence fusilade acts to a greater extent on actively growing plants. This result is in agreement with the hypothesized activity of fusilade in ATP synthesis, as actively growing plants require and produce more ATP than slow growing plants.

10.7. The effect of kerb on white clover *in vitro*.

10.7.1. Effect of kerb concentration and application time.

Untreated plants of the 3 week trial had greater fresh weight, dry weight and lateral root numbers than control plants of the 3 day trial, although untreated plants of the 3 day trial had significantly more leaves.

Kerb at both concentrations was more toxic to shoot height ($p<.001$), root length ($p<.001$), fresh weight ($p<.001$) (graph 5.1), dry weight ($p<.001$), lateral root numbers ($p<.001$), leaf numbers ($p<.001$), nodule numbers ($p<.001$) (graph 5.2) and acetylene reduction ($p<.01$) (graph 5.3) when applied to plants 3 days after germination than at 3 weeks.

10.7.2. Effect of nitrogen.

Untreated plants indicated that nitrogen normally had a significant effect on all parameters measured, increasing plant growth while inhibiting nodulation and nitrogenase activity. Kerb treated plants responded to nitrogen in the same way as controls, but to a lesser degree (graph 5.5), particularly when treated 3 days after germination (graph 5.4).

10.7.2.1. Effect of nitrogen and kerb concentration.

Plants grown on media supplemented with nitrogen were inhibited more by kerb as compared to controls, than were plants grown without nitrogen supplied (graph 5.5).

10.7.3. Effect of rhizobial inoculation.

Rhizobial inoculation significantly stimulated shoot height, root length, fresh weight, dry weight, leaf numbers and shoot fresh weight of untreated plants. Kerb at 10 x concentration inhibited the normal response of white clover plants to rhizobia in root length as plants treated 3 days after germination had no nodules. Plants treated 21 days after germination with 1 x concentration of kerb did show an increase in root length when inoculated, although plants treated with 10 x concentration of kerb 21 days after germination did not (graph 5.7).

10.7.3.1. Effect of rhizobial inoculation and application time.

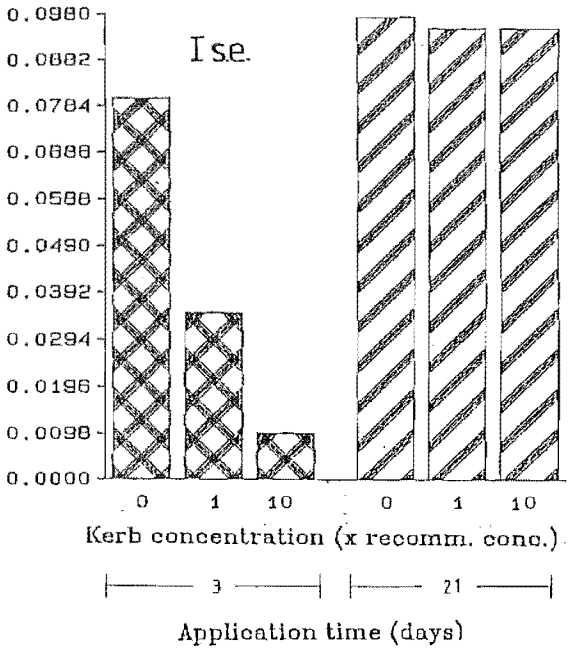
Plants treated with kerb 3 days after germination showed less stimulation of plant growth in response to rhizobial inoculation than plants treated 3 weeks after germination. This result is due to the complete lack of nodulation of plants treated with kerb 3 days after germination (graph 5.2).

10.7.3.2. Effect of rhizobial inoculation and kerb concentration.

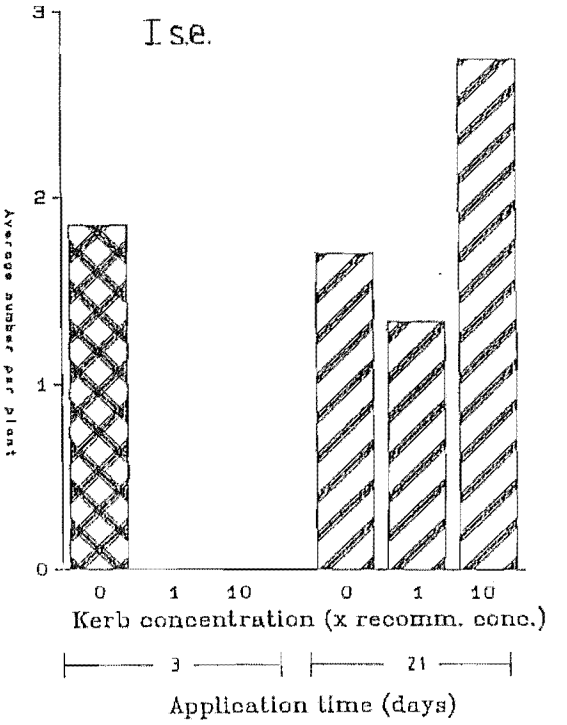
Plants treated with kerb 21 days after germination did responded normally to rhizobial inoculation (graph 5.6). Plants treated with 10x concentration of kerb did not show rhizobial stimulation of root length but responded normally for other parameters (graph 5.6 and 5.7). Kerb application at 10 x concentration inhibits rhizobial stimulation of root elongation.

Kerb

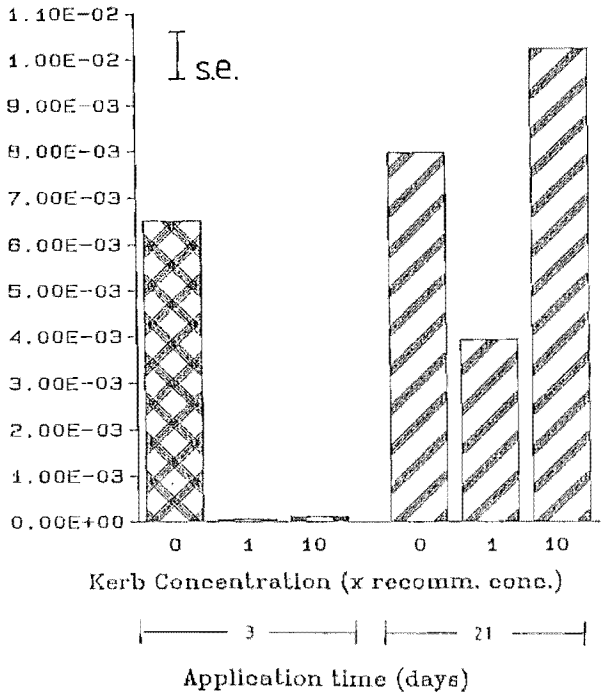
5.1. Effect on Fresh Weight.



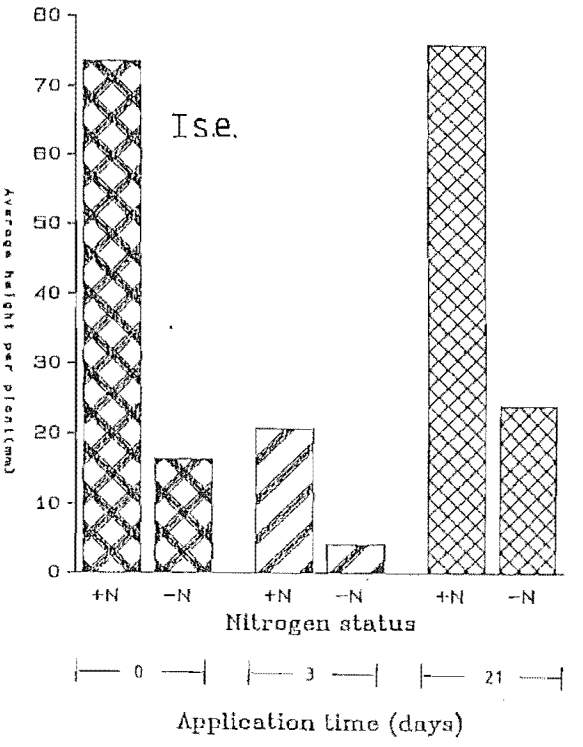
5.2. Effect on Nodulation.



5.3.Effect on Nitrogenase Activity.

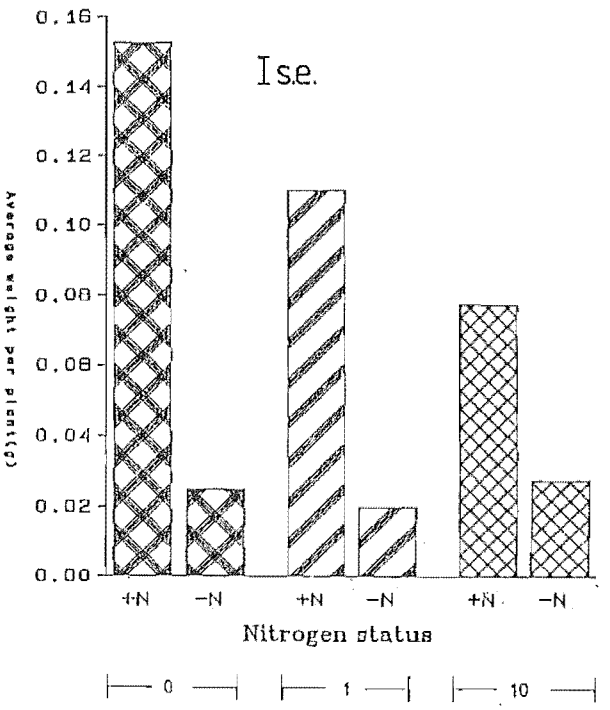


5.4.Influence of Nitrogen on Shoot Height.

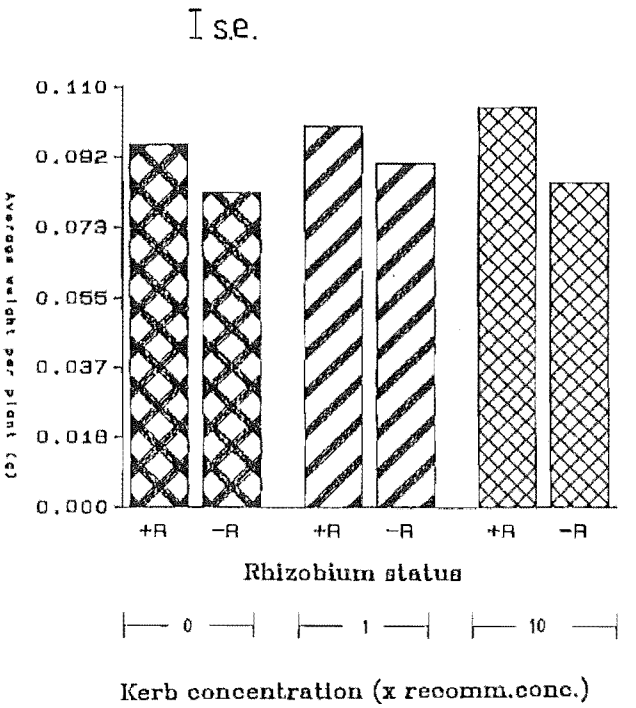


Kerb

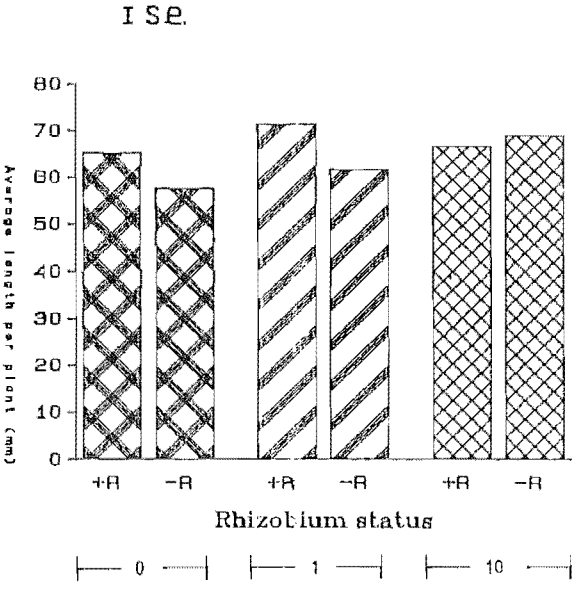
5.5.Influence of Nitrogen on Fresh Weight



5.6.Influence of Rhizobium on Fresh Weight.



5.7.Influence of Rhizobium on Root length.



10.7.4. Discussion of the effect of kerb on white clover *in vitro.*

Kerb is highly toxic to white clover, particularly at early application times and at high concentrations. The presence of an amide group within the side-chain of the compound, suggests that breakdown of the herbicide may stimulate plant growth by release of the bound nitrogen, and certainly plants lacking rhizobial inoculation did have greater fresh weight (graph 5.6) and root length (graph 5.7) when treated with kerb. Also 10 x concentration of kerb applied 21 days after germination did stimulate nodulation and nitrogenase activity (graphs 5.2 and 5.3). Carlson et al.(1975) reported that kerb has an intermediate persistence in soils remaining for 3-8 months, and is only gradually degraded by soil microorganisms. High levels of kerb may release small amounts of nitrogen from kerb through degradation by the rhizobial bacteria or chemical breakdown. Small amounts of nitrogen, particularly at early stages of growth, is known to stimulate nodule numbers at later stages due to early increase in plant weight (Richardson *et al.* 1957). This effect may account for the described stimulation. An *in vitro* system over this short time period apparently did not provide the conditions conducive to large scale deterioration of kerb.

Plants at the seedling stage were more susceptible to kerb toxicity than mature plants. Nodule numbers per plant were inhibited by both concentrations of kerb completely at the early application time. Kerb did not alter plant growth response to nitrogen in the medium (graph 5.5) although plants treated at the early seedling stage responded only partially, as compared to controls (graph 5.4). The mode of action of kerb has been reported as inhibition of cell division and growth (Carlson et al.1975). This is consistent with the present result, where kerb halted growth of white clover very rapidly following application. Plants grown on medium containing nitrogen were affected more by kerb than plants grown without nitrogen.

Nodulation and nitrogenase activity were inhibited more by 1 x concentration of kerb than by 10 x concentration when treated 21 days after germination. Kerb inhibited nodulation rather than nitrogenase activity, as decline in nitrogenase activity was directly proportional to decline in nodulation. Hence kerb appears to act specifically on formation of nodules, whether this be indirectly through an effect on the receptiveness of the plant to nodulation, or on some other factor required for nodule establishment.

Chapter 11.0. Results and Discussion of *In Vivo* Study of Herbicide Toxicity to *Trifolium repens* and its Symbiosis with *Rhizobium trifolii* RS102.

11.1. Preamble.

Pot experiments were designed to indicate the relevance and value of *in vitro* experimentation in determining herbicide toxicity. The emphasis of this section will therefore be on comparison of this experiment to *in vitro* results.

Domsch (1978) suggested that the interaction of pesticides with natural stress situations would be a problem worthy of attention. Humidity, temperature, pH and other soil factors were noted by Greaves and Malkomes (1980) as influences that could modify the response of organisms to pesticides. In the present experiment, the influence of the water content of the soil was, therefore, also investigated.

Results are tabulated in Appendix 4 and presented in graphs 6.1 to 10.3. Unless otherwise stated significance is $p<0.05$.

11.2. Controls.

Analysis of control pots within the experiment showed no significant difference between the controls allocated to each group of herbicide treated pots. Water level was a significant factor in all parameters measured. Plants grown in soil maintained at 50 % water holding capacity had significantly lower numbers of nodules ($p<.001$) (graph 6.2), shoot fresh weight ($p<.001$) (graph 7.1), root fresh weight ($p=.004$) and dry weight ($p=.002$) (graph 6.3). Higher water levels in the soil increase nutrient availability to plant roots, thereby increasing plant dry matter and growth.

Acetylene reduction values obtained from pot experiments did not correlate to any growth parameters in controls. This is probably due to the transfer of the pots to growth rooms 3 days before acetylene reduction in an effort to standardize environmental conditions prior to acetylene reduction and so avoid the effect of environmental variation on nitrogenase activity. However light intensity in the growth rooms used was later found to be approximately 10 % of normal daylight. This sudden decrease in light intensity is highly likely to inhibit nitrogenase activity (Sprent 1976). Hence acetylene reduction values for pot experiments were not included in further analysis.

11.3. The effect of paraquat on white clover *in vivo.*

11.3.1. Results.

Paraquat at 1 x concentration significantly lowered plant nodulation ($p=.02$) (graph 6.2), shoot weight ($p=.029$) and fresh weight ($p=.037$) (graph 6.1) and dry weight (graph 6.3). 10 x concentration of paraquat decreased ($p<.001$) all parameters measured (graph 6.1, 6.2 and 6.3).

Soil water level in pots significantly affected all growth parameters of paraquat treated plants. Plants grown in soil maintained at 50 % water holding capacity had significantly lower levels of fresh weight ($p<.002$) (graph 6.1), with both root ($p<.01$) and shoot weight ($p<.001$) being inhibited. Nodules per plant were also significantly inhibited ($p<.002$) by water levels of the soil (graph 6.2). This effect was identical to that observed among untreated plants and therefore paraquat did not alter the effect of water availability on plant growth.

11.3.2. Discussion.

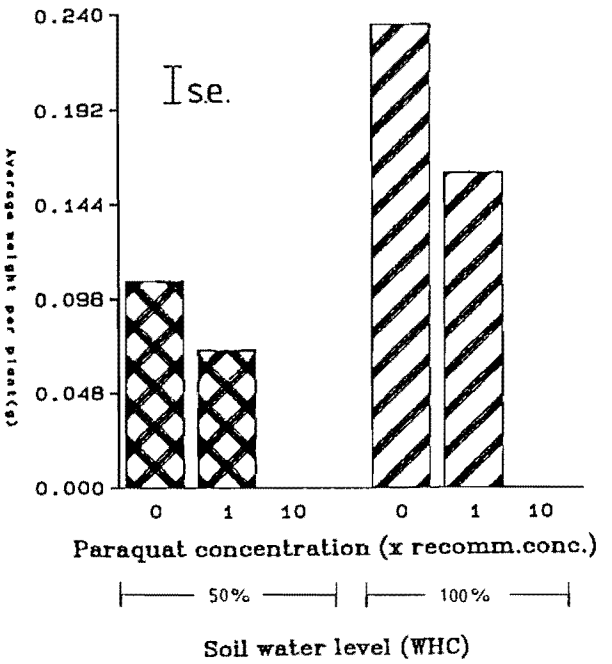
High concentrations of paraquat were extremely toxic toward white clover growth (graph 6.1). The effect of 1 x concentration of paraquat reflects paraquat's dessicant activity, with aerial plant parts being affected more than below ground parts. The response of clover plants to paraquat, particularly at 1 x concentration, was not as severe when treated in soil as when treated *in vitro*. Manninger *et al.* (1972) found paraquat applied to *Pisum sativum* in sand culture in a glasshouse inhibited yield and nodulation. However sand is not a very realistic medium in which to apply herbicides, particularly ionic ones such as paraquat.

Treatment of plants with paraquat did not alter the response of plant growth and nodulation to water regime, although high soil water did not convey as great an advantage on paraquat treated plants as on untreated plants (graph 6.1). High water levels in soil increase paraquat damage to plant nodulation and fresh weight as compared to treatment of plants grown in soil maintained at low water levels (graph 6.1 and 6.2). Paraquat is speedily inactivated in soil due to reactions between the double positively charged cations of the herbicide and negatively charged sites on clay minerals. Greater levels of soil water would decrease the amount of paraquat bound to soil colloids causing more paraquat to be available for plant contact.

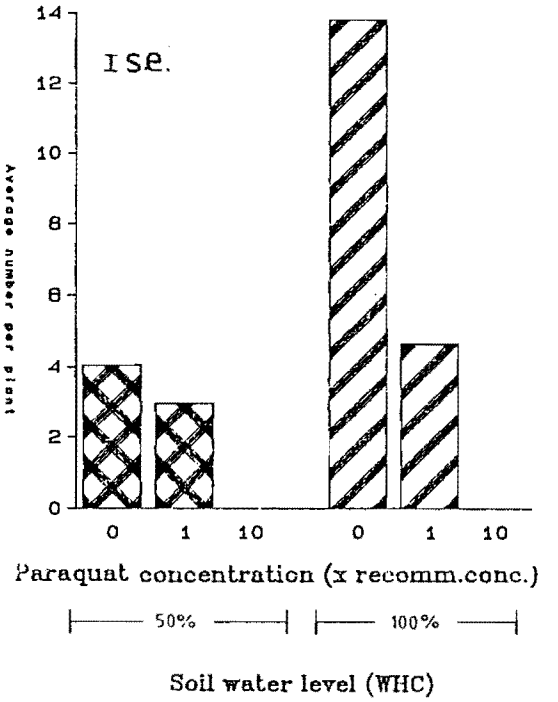
Root weight was not as affected following foliar application of paraquat to pots, therefore response of nodules to 1 x concentration of paraquat treatment may be due to loss of photosynthetic products, rather than a direct effect of the herbicide on nodulation. Peters and Ben Zbiba (1979) found nitrogen fixation appeared to be reduced mainly where the vigor and growth of the legume plants were reduced due to effects of the herbicide. Paraquat is known to be translocated in the apoplastic system. Garcia and Jordan (1969) suggested that translocation of 2,4DB in *Lotus corniculatus* may concentrate pesticides in specific regions of the plant and thereby reach nodules at dangerous levels. Hence it is possible that nodule activity could be affected by paraquat directly, particularly when exposed to high concentrations of the herbicide.

Paraquat

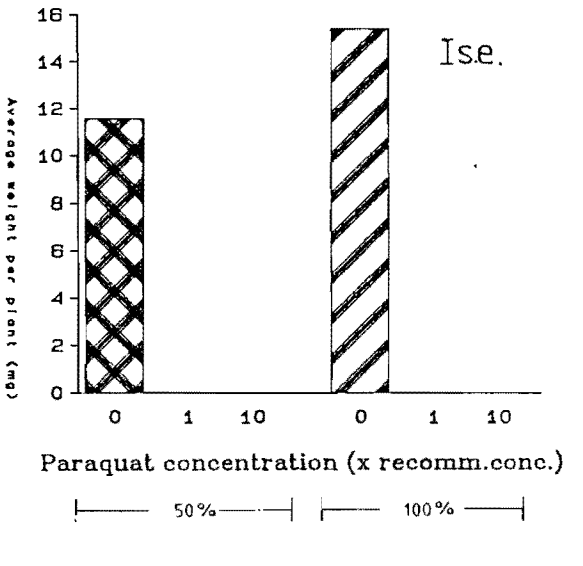
6.1.Effect on Plant Total Fresh Weight



6.2.Effect on Plant Nodulation



6.3.Effect on Plant Total Dry Weight.



Blood (1962) found that utilizing paraquat to manage the ratio of grass to white clover in a sward reduced clover yield, but did not affect the viability of seed produced.

11.4. The effect of MCPB on white clover *in vivo.*

11.4.1. Results.

MCPB at 1 x concentration inhibited shoot fresh weight (p=.019) (graph 7.1), average plant fresh weight (p=.023) (graph 7.2), nodule numbers (graph 7.4), plant dry weight (graph 7.3) and fresh root weight were unaffected.

10 x concentration of MCPB significantly lowered dry weight (p=.001) (graph 7.3), shoot fresh weight (p<.001) (graph 7.1) and total fresh weight (p<.001) (graph 7.2) of treated plants. Nodule numbers were significantly less in plants treated with high levels of MCPB (graph 7.4). Root weight of plants was not significantly inhibited by high levels of MCPB.

Water regime was a significant factor (p<.03) in the level of all parameters of plants treated with MCPB at either application rate (graph 7.1 & 7.4). Plants grown in soil having less available water were significantly inhibited as compared to those grown in soil maintained at maximum water holding capacity, that is, MCPB treated plants responded normally to soil water level. Fresh weight of plants grown in soil maintained at maximum water holding capacity was inhibited more by MCPB at high levels than plants grown in dryer soil (graph 7.1 & 7.2)

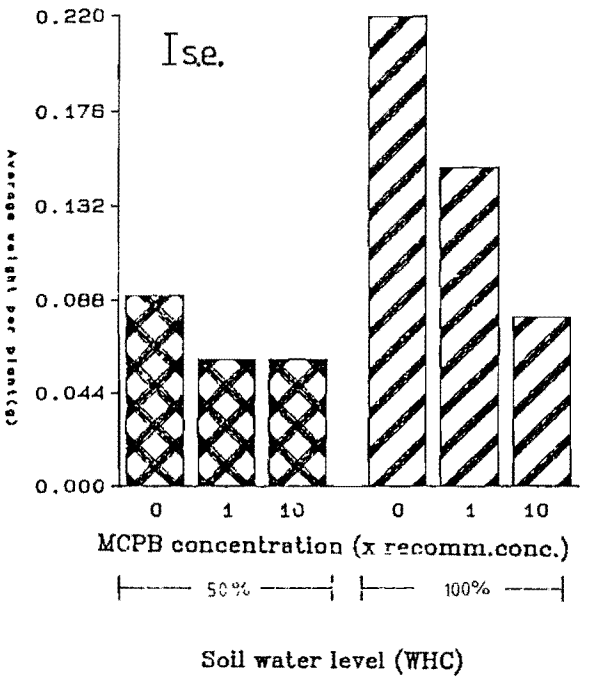
11.4.2. Discussion.

10 x concentration of MCPB affected dry as well as fresh weight of white clover plants, (graph 7.3 and 7.2) as in *in vitro* experiments. 1 x concentration of MCPB acted primarily on aerial parts of the plant, as shown by inhibition of shoot fresh weight (graph 7.1), unlike *in vitro* experiments, where 1 x concentration of MCPB showed an affect on both aerial and below ground plant parts. This difference is probably due to MCPB applications *in vitro* being made to the whole plant, whereas *in vivo* MCPB was applied foliarly.

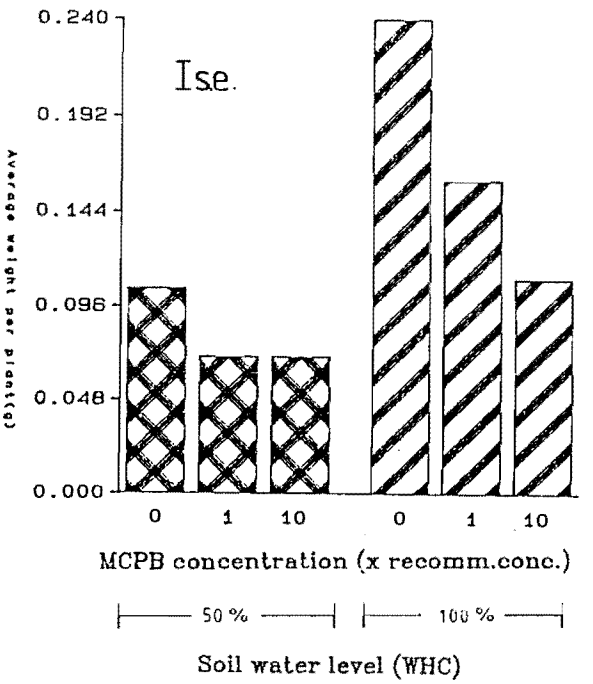
Plants treated with MCPB in pots at both concentrations exhibited identical root deformation as plants treated *in vitro* (color plate 1). MCPA is known to produce callus growth and root primordia on mature parts of roots. Roots thereby lose the ability to take up nutrients and photosynthesis is reduced (Audus 1964). 1 x concentration of MCPB appears to inhibit fluid translocation to aerial plant parts, as shoot fresh weight (graph 7.1) and total fresh weight (graph 7.2) are inhibited, although total dry weight (graph 7.3) and root fresh weight do not show any effect. *In vitro*, MCPB at 1 x concentration inhibited root length and total plant dry weight. Shoot and root fresh weight were not measured separately *in vitro*, hence a comparison of the response of these parameters cannot be made. However *in vitro* MCPB appears to give a more toxic effect than in pots, as dry weight of plants was affected by 1 x concentration of MCPB in plate experiments.

High concentrations of MCPB *in vivo* exhibit greater toxicity than 1 x concentration. Dry weight as well as fresh weight was lowered (graph 7.3), with shoot fresh weight but not root fresh weight being inhibited (graph 7.1). As MCPB in its

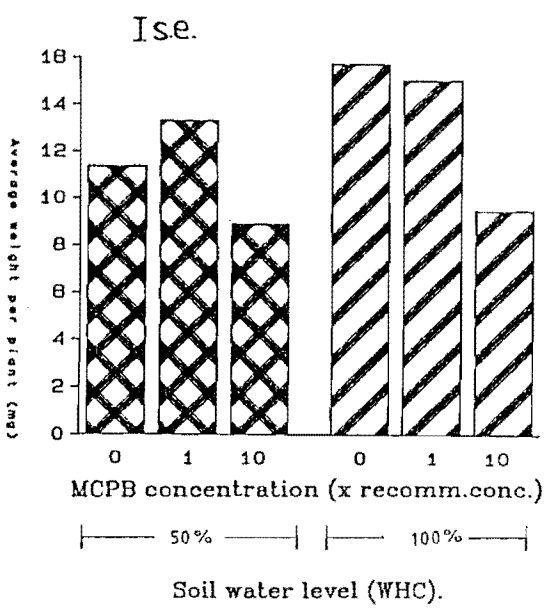
7.1.Effect on Plant Shoot Fresh Weight.



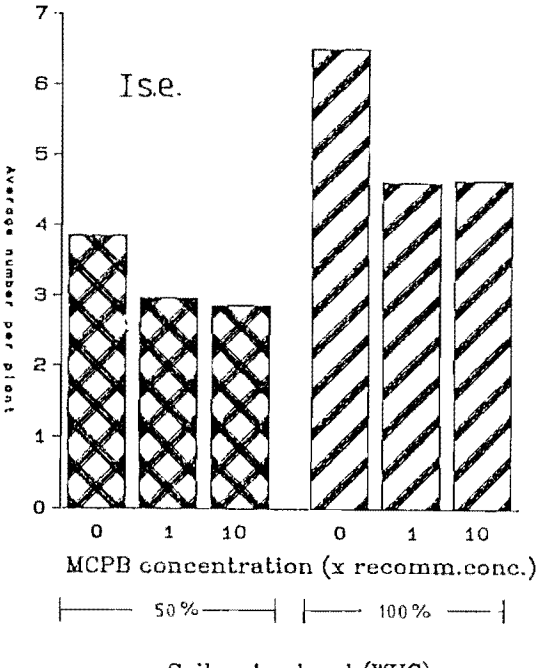
7.2.Effect on Plant Total Fresh Weigh



7.3.Effect on Plant Total Dry Weight.



7.4.Effect on Plant Nodulation.



active state causes root deformation and loss of ability to take up nutrients, a decline in plant dry matter is consistent with this activity.

Root fresh weight was unaltered. MCPA causes formation of callus tissue on roots, which appears to have a high water content, hence root fresh weight does not appear altered although roots are severely deformed. Nodulation was significantly less in plants treated with MCPB *in vivo*. Nodulation did not show significant inhibition by this herbicide *in vitro*, although in all cases nodulation was less than controls. Identification of nodules was a serious problem in MCPB treated plants due to severe root deformation caused by the herbicide. It is probable that MCPB treatment could have reduced nodule numbers as infection sites on roots of the host plant by *Rhizobium* species were probably reduced. A similar effect was found in *Vigna sinensis* and *Centrosema pubescens* treated with phenylurea herbicides (Amakiri and Odu 1978).

A direct effect of the herbicide on *R.trifolii* is unlikely as although growth of this bacterium *in vitro* was affected by MCPB when applied on discs or in wells, growth in liquid culture was unaffected. Brock (1972) found that nodules per plant and root dry weight were positively correlated, and nodules per unit root dry weight were relatively constant at all rates, therefore it was concluded that the herbicides tested did not have a direct effect on the rhizobial population or on its ability to form nodules.

Water level affected growth of MCPB treated plants in an identical manner to untreated plants, hence MCPB did not alter plant response to water availability. Plants grown in soil maintained at maximum water holding capacity were inhibited more by 10 x concentration of MCPB than plants grown in soil maintained at 50 % water holding capacity (graph 7.1 & 7.2). Plants grown in soil with a high level of water would have a greater availability of nutrients and, as MCPA has greater effect on rapidly growing individuals having a good nutritional status, it would be expected that these plants are affected to a greater extent. Alternatively the higher soil water level may cause the herbicide to be more likely to contact the plant, as appeared to occur with paraquat. *In vitro* experiments showed plants grown on medium containing nitrogen were more inhibited by MCPB than plants lacking a nitrogen supply. It seems probable from the effects of this herbicide that the MCPB distributed by the manufacturers contains some MCPA or breakdown of MCPB to its active form is occurring as white clover has been shown to be resistant to pure MCPB (Fletcher *et al.*1956).

11.5. The effect of bentazone on white clover *in vivo.*

11.5.1. Results.

Recommended levels of bentazone had a significant inhibitory effect on shoot fresh weight (p=.033), plant total fresh weight (p=.041) (graph 8.1) and nodulation (p=.010) (graph 8.2). Plant root fresh weight and dry weight were unaffected by bentazone at 1 x concentration under high soil water.

10 x concentration of bentazone significantly inhibited shoot fresh weight (p<.001), plant total fresh weight (p<.001) (graph 8.1) and nodulation (p<.001) (graph 8.2) to a greater extent than recommended levels of bentazone.

Water level significantly affected (p<.02) all growth parameters of plants treated with recommended levels of bentazone. Plants grown in soil maintained at 50 % water holding capacity had significantly lower levels of nodules (p<.001) (graph 8.2), shoot fresh weight (p<.001), root fresh weight (p=.001) and total fresh weight (p<.001) (graph 8.1) when treated with 10 x concentration of bentazone than controls. Dry weight of plants treated with bentazone at 1 x concentration was significantly affected by soil water level although dry weight of plants treated with 10 x concentration of bentazone was not (graph 8.3). 1 x concentration of bentazone did not affect root weight and dry weight of plants grown in soil maintained at maximum water holding capacity, whereas these parameters were significantly inhibited by 1 x concentration of bentazone when plants were grown in soil maintained at 50 % water holding capacity.

11.5.2. Discussion.

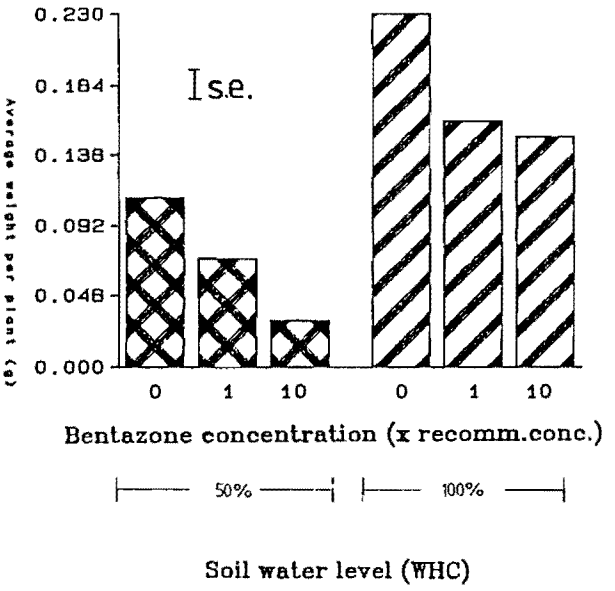
Bentazone at 1 x concentration caused significant decreases in shoot growth and nodulation (graph 8.2). 10 x concentration of bentazone exhibited similar toxicity to white clover growth as 1 x concentration, but was more severe (graph 8.1). Ljjungren and Martensson (1980) found bentazone had a mild effect up to 8 ppm, lowering numbers of root hairs infected. 1 x concentration (approximately 1-2ppm), foliarly applied, caused deformation of root hairs in soil and ineffective nodule formation.

In vitro testing showed bentazone to be non-toxic to white clover plants. Interactions between a particular chemical and the soil environment may alter the chemical to a toxic form. In pot experiments soil was sterilized, however sterility is unlikely to remain over the 4 weeks of the experiment in a glasshouse therefore abiotic changes may occur. For example, Webley *et al.*(1958) found *B*-oxidation of side-chains of phenoxybutyric acids was carried out by soil Nocardias, which converted these herbicides to their active form. Fisher *et al.*(1978) found correlation between tests *in vitro* and *in vivo* was poor. This author compared *in vitro* experiments of pesticide toxicity to bacteria with *in vivo* experiments on plant growth, as these experiments are on different organisms under different conditions variation in results would not be unexpected.

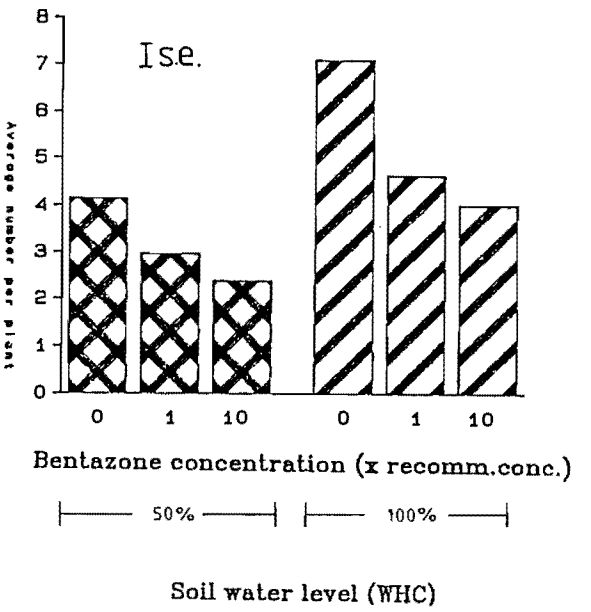
Skuterad and Caseley (1980) found no transport of bentazone or its metabolites could be detected in *Glycine max* (soybean) following foliar application

Bentazone

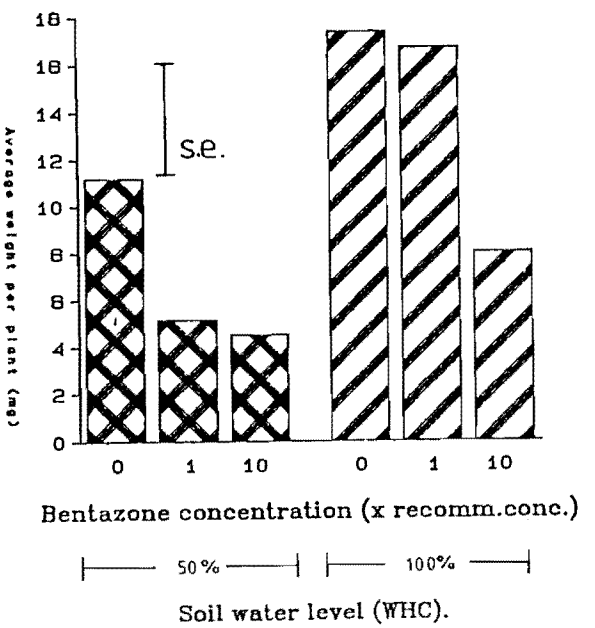
8.1.Effect on Plant Total Fresh weight.



8.2.Effect on Plant Nodulation.



8.3.Effect on Plant Total Dry Weight.



although uptake was achieved via roots from the soil. Root fresh weight did not show any response to bentazone in the present *in vivo* experiment following foliar application, indicating that bentazone probably does not affect nodulation by translocation through root tissue from soil. However shoot tissue did show an inhibition in growth, hence white clover does appear to take up bentazone through the leaves or bentazone was taken up by roots but acted only on the foliage of the plant. It seems probable therefore, that damage to nodules is due in part to loss of photosynthates from damaged foliage rather than a direct effect on the nodules. *In vitro* application of bentazone to white clover often showed a stimulatory affect on root development. As application of herbicides was made to both shoot and root in plate experiments, root uptake is more probable *in vitro* than under *in vivo* conditions where herbicides were foliarly applied.

10 x concentration of bentazone stimulated root fresh weight of plants grown in soil maintained at maximum water holding capacity, but inhibited these parameters in plants grown in soil maintained at 50 % water holding capacity. Bentazone is not persistent in soil, 5 ppm applied to a sandy loam broke down within 15 weeks (BASF technical communication). A bentazone molecule contains 2 N atoms. Skuterad and Caseley (1980) found bentazone absorption through roots was greater under wet conditions, hence the herbicide exhibited greater toxic effects when followed by rain. It is possible that with high levels of soil water bentazone deterioration is more rapid, allowing small amounts of bound nitrogen to be released and thereby becoming available for plant uptake. Alternatively as suggested by Diatloff (1970) good moisture conditions may mitigate potentially toxic effects of pesticides by the dilution of the chemical.

11.6. The effect of fusilade on white clover *in vivo.*

11.6.1. Results.

Fusilade at recommended levels significantly affected nodulation (p=.025) (graph 9.1) and plant fresh weight (graph 9.2). 10 x concentration of fusilade significantly inhibited root fresh weight (p=.004), shoot fresh weight (p=.001), total plant fresh weight (p=.001) (graph 9.2) and nodulation (p=.002) (graph 9.1).

Plants grown in soil maintained at 50 % water holding capacity had significantly lower levels of shoot fresh weight (p<.001), root fresh weight (p=.025) and total fresh weight (p<.001) (graph 9.2) when treated with fusilade at 1 x concentration. However dry weight was not significantly inhibited by 1 x concentration of fusilade. Dry weight of plants was not affected by fusilade at either concentration when soil was maintained at 100 % water holding capacity (graph 9.3).

11.6.2. Discussion.

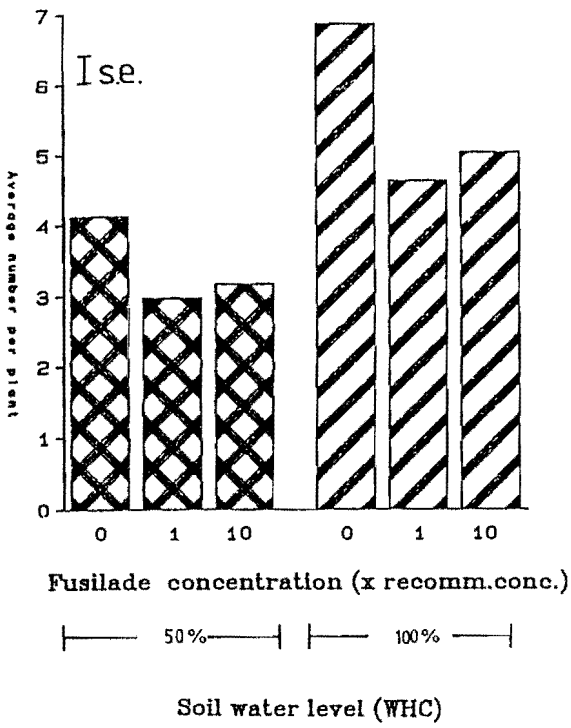
1 x concentration of fusilade lowered nodulation and fresh weight, but did not affect dry weight of white clover plants. Plants grown in pots would be using available nitrogen in the soil during early development therefore nodules may not form until the second or third week after planting. Plants were treated with herbicides 25 days after planting, therefore herbicides would be contacting plants during nodule development. Any inhibition of growth due to lowered nodulation such as lowered dry weight may not be detected until later in development as soil nitrogen becomes depleted.

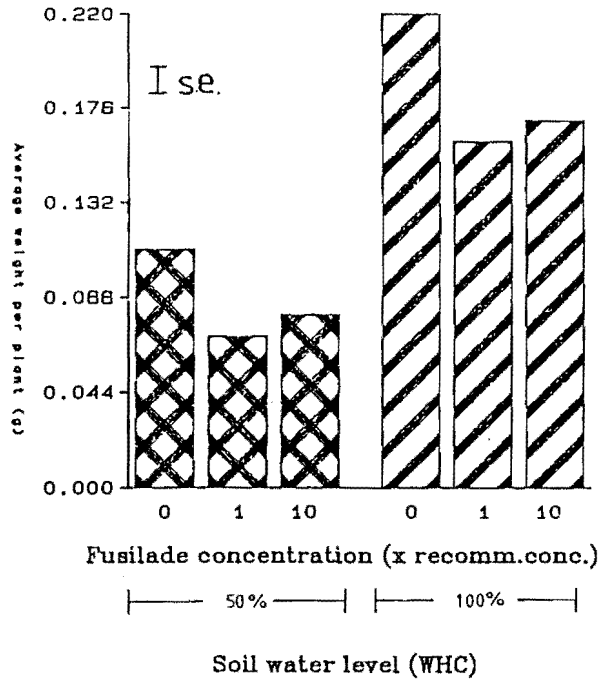
Fusilade at 1 x concentration did not affect plant dry weight (graph 9.3). *In vitro* experiments showed 10 x concentration of fusilade inhibited all growth parameters except nodulation (graph 4.2), while 1 x concentration of this herbicide had an identical, but not as pronounced an effect. Hence *in vivo* applications of fusilade showed a greater affect on nodule numbers (graph 9.1), but *in vitro* application tended to act against plant growth and nitrogenase activity.

Fusilade effectiveness has been found to be altered by drought stress or low humidity (Plowman *et al.*1980). Plants treated with 1 x concentration of fusilade and grown in soil maintained at maximum water holding capacity did not have lower dry weight (graph 9.3), although nodule numbers were significantly lower (graph 9.1). Hence although plant growth did not show loss of dry matter, nodulation was susceptible to fusilade treatment. *In vitro* experiments showed fusilade at both concentrations did not affect nodule number, but did affect nitrogenase activity and plant growth. *In vitro* testing of fusilade toxicity to *R.trifolii* showed no growth inhibition of the bacterium by this herbicide. Hence effects on nodulation are via toxicity to plant contribution to nodulation and not through damage to the bacteria.

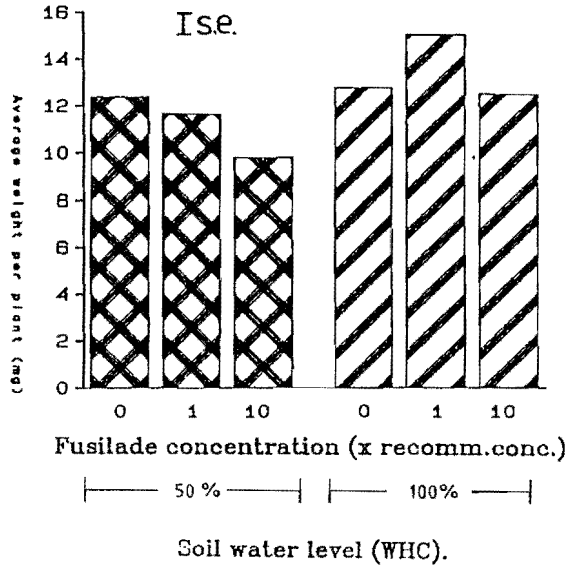
Plowman *et al.*(1980) found growth of susceptible plant species was halted rapidly by fusilade, but existing leaves are slow to die. The speed of action was accelerated in some species by higher rates of application. Fusilade at high concentration did significantly affect white clover growth *in vivo* and *in vitro*. All fresh

9.1.Effect on Plant Nodulation. 9.2.Effect on Plant Total Fresh weight.





9.3.Effect on Plant Total Dry Weight.



weight parameters were significantly lower in plants treated with 10 x concentration of fusilade, but dry weight was not affected. Fusilade at high concentrations appears to exert a dessicant type activity. Fusilade is known to rapidly translocate in both the phloem and the xylem (Plowman et al.1980) hence it affects both shoot and root tissue. The present study also indicated toxicity of fusilade to both shoot and root tissue as both shoot and root fresh weight was affected by fusilade treatment.

Nodulation was significantly inhibited by fusilade at 10 x concentration (graph 9.1). It is possible this inhibition may be directly on the nodules, as fusilade is readily translocated, and root development was also affected. The effect of this herbicide applied directly to foliar parts of the plant could be compared to the effect when applied to plant roots. This would determine whether the herbicide was translocated to nodules, or acted via loss of photosynthetic products.

11.7. The effect of kerb on white clover *in vivo.*

11.7.1. Results.

Kerb at 1 x concentration significantly inhibited nodulation (p=.03) (graph 10.1), shoot fresh weight (p=.035) and total plant fresh weight (p=.038) (graph 10.2). Root fresh weight and plant dry weight were unaffected by normal applications of kerb at high soil water levels (graph 10.3). High levels of kerb did not significantly affect any parameter.

Kerb treated plants grown in soil maintained at 50 % water holding capacity had significantly lower levels of nodules (p=.006) (graph 10.1), shoot weight (p<.001), root weight (pp=.018) and total plant fresh weight (p<.001) (graph 10.2) than plants grown in soil maintained at maximum water holding capacity. However dry weight of kerb treated plants was not affected by water availability (graph 10.3), as occurred in controls.

When treated with kerb at 10 x concentration, low water availability significantly inhibited nodulation (p<.001) (graph 10.1), shoot weight (p<.001), root weight (p=.003) and total plant fresh weight (p<.001) (graph 10.2), as was found in controls.

11.7.2. Discussion.

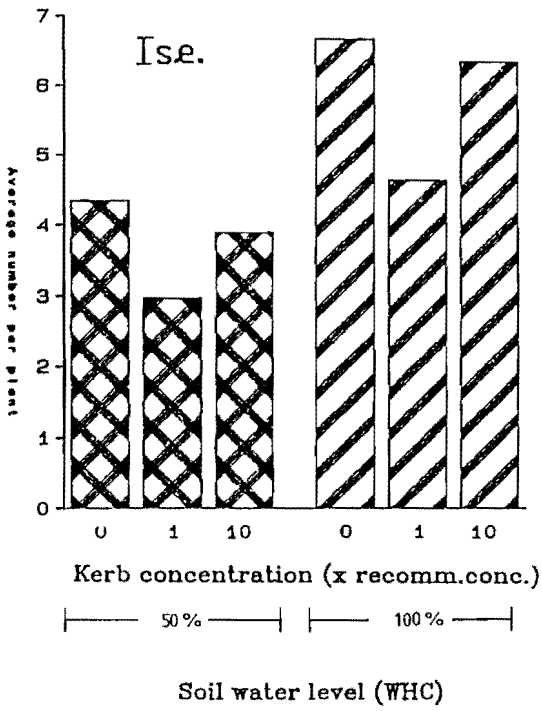
Kerb at 1 x concentration exhibited greater toxicity to white clover plants than this herbicide caused at 10 x concentration (graph 10.2). Kerb is a substituted amide herbicide. It is possible that any toxicity of this herbicide at 10 x concentration was masked by the input of nitrogen caused by release of the amide side-chain. Dunigan *et al.*(1972) found low and medium rates of 2 herbicides applied to soybean in field trials (see table 4) caused detrimental effects, while some of the higher rates of these herbicides did not, these authors did not provide an explanation for their result.

1 x concentration of kerb significantly lowered shoot fresh weight and total plant fresh weight (graph 10.2), although root fresh weight and total dry weight were not altered under high soil water conditions as compared to controls (graph 10.3). This result indicates a possible dessicant activity of kerb at this concentration. Kerb is believed to be readily absorbed by roots and translocated upwards and distributed throughout the plant. Translocation from leaves is not appreciable (Carlson *et al.*1975). Nodulation was significantly inhibited by 1 x concentration of kerb, but showed no response to 10 x concentration of kerb (graph 10.1). Therefore it seems inhibition of nodulation caused by kerb is a reflection of plant growth response and not a direct effect of the herbicide on nodulation. This herbicide showed no effect against plant root growth, indicating that damage occurred only to the aerial plant parts.

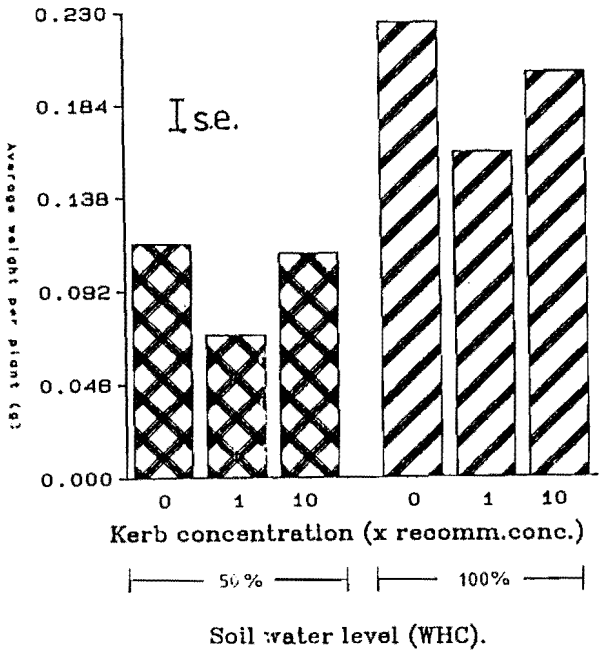
Yih *et al.*(1970) found activity of kerb under hot dry conditions was inferior to that under cool moist conditions, as breakdown in soil is much faster under high temperatures by chemical degradation. In the present experiment water content of the soil was found to have no effect on activity of kerb toward white clover growth or nodulation (graph 10.1 and 10.2).

Kerb

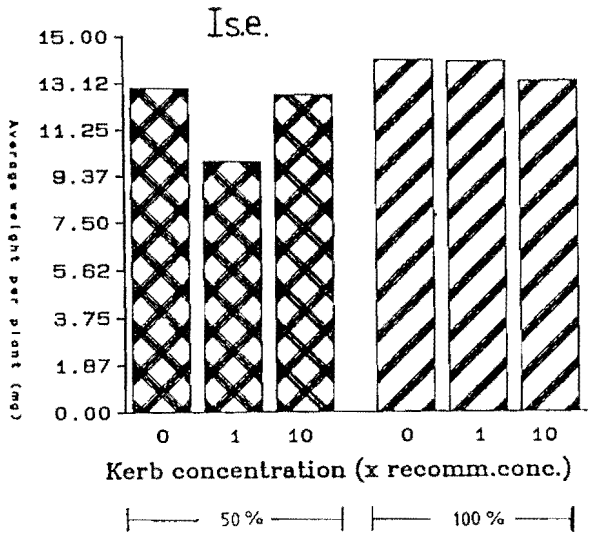
10.1.Effect on Plant Nodulation.



10.2.Effect on Plant Total Fresh Weight.



10.3.Effect on Plant Total Dry Weight.



In vitro experiments showed kerb to be extremely toxic to white clover growth at both concentrations used. The lower degree of toxicity of kerb shown in pot experiments (graph 10.2), and the lack of effect of 10 x concentration of kerb must be related to an interaction of kerb with soil. Carlson *et al.*(1975) reported that kerb has an intermediate persistance in soils, and is gradually degraded by soil microorganisms. Hence it is possible that *in vivo* kerb is being degraded, thereby exerting less toxicity than *in vitro*. Garcia and Jordan (1969) found the degree of herbicide effect was more severe in growth chambers than in the field because of the absence of interacting factors which might have reduced the phytotoxicity of the herbicide. Under aseptic conditions the complicating effects of soil biotic factors were absent.

Chapter 12.0. General Discussion of Toxicity of Herbicides Toward White Clover.

12.1. Effects on *R.trifolii* RS102 *in vitro*.

In vitro testing in liquid medium indicated that the growth of the bacteria *Rhizobium trifolii* was unaffected by the herbicides under test. However this does not eliminate the possibility that the potential of the bacteria to nodulate white clover is affected. Research by other workers (eg. Grossbard 1970b) does indicate that if herbicides damage a symbiosis it is via the plant and not through an effect on the bacteria, although Fletcher and Raymond (1956) found sub-bacteriostatic doses of phenoxyherbicides reduced the ability of *R.trifolii* to form a symbiosis with clover.

12.2. Effects on *Trifolium repens* (white clover) and its symbiosis with *R.trifolii*.

In vitro experiments showed white clover was very sensitive to herbicides when contacted at the early seedling stage. This indicates the possible danger of planting white clover seed after a previous crop has been chemically treated or cleared with herbicides.

The effects found in experiments of herbicide toxicity to non-target microoganisms may either be due to the active ingredient of the herbicide mixture or to other components added to the active ingredient to aid in herbicide activity or ease of application. As the herbicides tested are used in agriculture in a formulated state it is of primary interest to determine if the herbicide is of toxicity in its used form. Whether toxicity is due to the active ingredient or added materials may be later determined.

Throughout the pot experiments the effect of all herbicides on nodulation mirrored the effect of the herbicide on plant growth parameters (graph 6.1 to 10.3). This similarity in response strongly implies that effects of herbicides on nodulation under *in vivo* conditions is due to loss of photosynthetic products from the plant rather than a direct effect of the herbicides on nodulation. *In vitro* this relationship was not as obvious, with nitrogenase activity and nodulation often varying independant of the plants growth response. As herbicides *in vitro* were applied to both the root and shoot, as compared to foliar application to plants in pots, it is probable that a more direct effect of the herbicides on nodulation would occur *in vitro*.

12.2.1. Paraquat.

Paraquat had severe effects on growth and nodulation of white clover both *in vitro* and in pot experiments. This herbicide was more toxic toward white clover *in vitro*, possibly due to the known affinity of paraquat to soil colloids, thereby rendering the herbicide inactive. Paraquat interfered with nodulation directly following treatment on agar, and acted rapidly to dessicate plant foliage, particularly at 10 x the

recommended concentration. However in pot experiments paraquat appeared to affect nodulation via loss of photosynthetic products, as root weight was not affected by the treatment while foliage was severely damaged. It has been suggested (Garcia and Jordan 1969) that translocation of a herbicide may concentrate pesticides in specific regions of the plant and thereby reach nodules at dangerous levels, this effect may be occurring following paraquat treatment. However the effect of paraquat on chloroplasts is well documented (Harris and Dodge 1972b) and it is probable that damage to nodules is to some degree via loss of photosynthetic products.

12.2.2. MCPB.

MCPB caused symptoms on plants both *in vitro* and in pots similar to those reported for the active form of this herbicide - MCPA (Gorter and Zweep 1964), particularly stunting root growth. This herbicide appeared to act on meristematic tissue of roots and to a greater degree on plants of higher nutritional status. MCPB caused a decrease in plant biomass but an increase in water content. Also, the presence of nitrogen failed to stimulate plant growth of MCPB treated plants which is in agreement with the previously reported activity of MCPA in causing roots to lose the ability to take up nutrients (Gorter and Zweep 1964).

MCPB treatment of plants in pots acted to a greater extent on aerial plant parts as compared to *in vitro* application, where MCPB affected mainly root tissue. This may be a reflection of the different application methods used as MCPB was foliarly applied to plants in pots whereas *in vitro* the whole plant was treated. Alternatively the reaction of plants in pots to MCPB was a dessicant one, with aerial plant parts suffering a loss of fresh weight and a not so severe loss of dry weight. This effect did not occur *in vitro* as plates were kept at high levels of humidity, and therefore plant leaves did not become dessicated.

Pot treatment of plants with MCPB did cause similar root deformation as was observed in plate experiments. Nodulation was lowered by both concentrations of MCPB *in vitro*. Nodulation of white clover was lowered by MCPB at the 10 x concentration in pots, although 1 x concentration did not significantly affect nodulation. Root deformation caused by MCPB may have reduced available infection sites as was suggested may occur by Amakiri and Odu (1978) for *Vigna sinensis* and *Centrosema pubescens* treated with phenylurea herbicides.

MCPB had a greater effect on plants grown in soil maintained at maximum water holding capacity, as compared to those grown in soil at 50 % water holding capacity. This may be due to higher soil water levels making nutrients more available to plants and MCPB therefore acts more severely on these plants due to their higher nutritional status, as seen *in vitro*. Alternatively higher soil water may cause higher levels of the herbicide in the soil solution, as appeared to occur with paraquat.

12.2.3. Bentazone.

Bentazone did not cause any severe inhibition of white clover growth *in vitro*, although this herbicide did show some inhibition of rhizobial plant growth stimulation at high concentrations. *In vitro* experiments indicated that bentazone may be affecting plant growth and nodulation due to breakdown of the herbicide releasing bound nitrogen. Application of bentazone to white clover in pots caused more severe inhibition of shoot growth and nodulation than *in vitro* experimentation indicated. It is possible that interactions between the herbicide and the soil environment enhance its toxicity, as found by Webley *et al.*(1958) for phenoxybutyric acid herbicides.

Bentazone at 1 x concentration did not inhibit dry weight of plants grown in soil maintained at 100% water holding capacity, although this level of bentazone did severely lower dry weight of plants grown in soil at low moisture levels. Inhibition of growth by this herbicide therefore appears to be associated with humidity, as *in vitro* experiments were also kept at high humidity, this herbicide may break down faster under wet conditions. Alternatively toxicity of bentazone may be mitigated under high moisture conditions by dilution of the herbicide.

12.2.4. Fusilade.

Fusilade was toxic to white clover *in vitro* when applied at high concentrations or shortly before harvest. Fusilade was particularly active against nitrogenase activity, as indicated by acetylene reduction and the lack of stimulation of growth caused by inoculation of fusilade treated plants with rhizobia. However nodule numbers were not lowered by fusilade *in vitro*, indicating that this herbicide acted directly against the nitrogenase enzyme. Fusilade also acted to a greater extent against vigorously growing plants. Fusilade is thought to adversely interfere with ATP production, as nitrogenase requires high levels of ATP to function (Dilworth and Glenn 1984) it is likely that fusilade inhibits nitrogenase activity by limiting the availability of ATP to the nodule.

In pots fusilade at 1 x concentration affected nodulation of white clover, while at 10 x concentration fusilade inhibited nodulation and plant weight. Hence *in vivo* application of fusilade showed a greater toxicity against nodulation, but *in vitro* fusilade inhibited plant growth to a greater degree. This difference may be due to the method of application of the herbicide, as *in vitro* herbicides were applied to the root as well as to the shoot of the plants, while *in vivo* applications were foliar. Both methods of testing indicated that nodules are particularly susceptible to fusilade activity.

12.2.5. Kerb.

Kerb was severely toxic to white clover growth and nodulation when applied *in vitro*. The effect of this herbicide on nitrogenase was via an effect on nodule numbers, and not a direct effect on nitrogenase activity, as found with fusilade treatment. However certain *in vitro* kerb treatments enhanced white clover growth, for example 10 x concentration of kerb 21 days after germination stimulated nodulation and nitrogenase activity. Kerb is an amide herbicide and may release small amounts of

nitrogen, which may explain this growth stimulation. Small amounts of nitrogen may stimulate nodulation via enhanced plant growth (Richardson *et al.*1957). The results of *in vitro* testing of kerb against white clover implied that this herbicide acted in a similar way to its effect on target plants, ie. through inhibition of cell division and growth (Carlson *et al.*1975).

In pots kerb exhibited greater toxicity to white clover at the recommended level than at 10 x this concentration. Kerb may be degenerating and releasing nitrogen, as the higher concentration would be expected to release proportionally more nitrogen than the 1 x concentration. This release of nitrogen may cause a growth stimulation masking any negative effects of the herbicide.

The response of nodulation to kerb treatment in pots mirrored that of the plant growth, indicating that, as for *in vivo* experiments, nodulation was affected by a loss of photosynthetic products, and not by a direct effect on the nodules.

Kerb was much less toxic to white clover in pots than *in vitro*, which may be because of the different application methods used, as previously discussed. Translocation of kerb from leaves was found to be not appreciable by Carlson *et al.*(1975), while it is believed to be readily absorbed by roots. Alternatively contact of kerb with the soil may alter kerbs activity, either by binding to soil particles, or degradation by chemical means or microorganisms. Garcia and Jordan (1969) found the degree of herbicide effect on non-target plants was more severe in growth chambers than in the field, because of the absence of interacting factors which may reduce herbicide phytotoxicity.

SECTION B:

HERBICIDE EFFECTS ON WHITE CLOVER NODULE ULTRASTRUCTURE

Chapter 13.0. Cytology and Ultrastructure of *Trifolium repens* (white clover) / *Rhizobium trifolii* Nodules.

13.1. Introduction.

This study was designed to determine the effects of pesticides on nodulation and plant growth.Ultrastructural studies of nodules from treated plants may provide information about the cellular processes that are disrupted by pesticide treatment, and the targets of pesticide activity within the cell. Also ultrastructural variation together with physiological information might indicate which processes are critical to nodule development and function.

Nodulation involves a very complex and delicate relationship which is likely to be easily disrupted by deleterious environmental effects. These effects may not be detectable above the ultrastructural level.

13.2. Legume nodule morphology.

13.2.1. Preamble.

To be able to identify and interpret a change caused by an environmental effect, what is "normal" in the structure must first be understood. Many studies of ultrastructure of symbiotic legume/rhizobial nodules have been undertaken. The bulk of these concentrate on soybean (*Glycine max*) nodules due to the importance of this crop economically and agriculturally.

Scattered studies of ultrastructure of other legume nodules have been published. Reviews of the subject (eg.Dart 1974; Bergersen 1982) tend to base descriptions of nodule ultrastructure on a range of studies of different nodules and thereby build up a picture of the "typical" nodule. The review given here by necessity uses a variety of sources, but attempts to base the description primarily on those few studies of *Trifolium repens/Rhizobium trifolii* nodules which have been carried out.

The spatial disposition adopted by the dividing cells of the nodule meristem cause the nodules of the various legumes to differ in shape, gross anatomy and fine structure. Two broad classes are recognized;

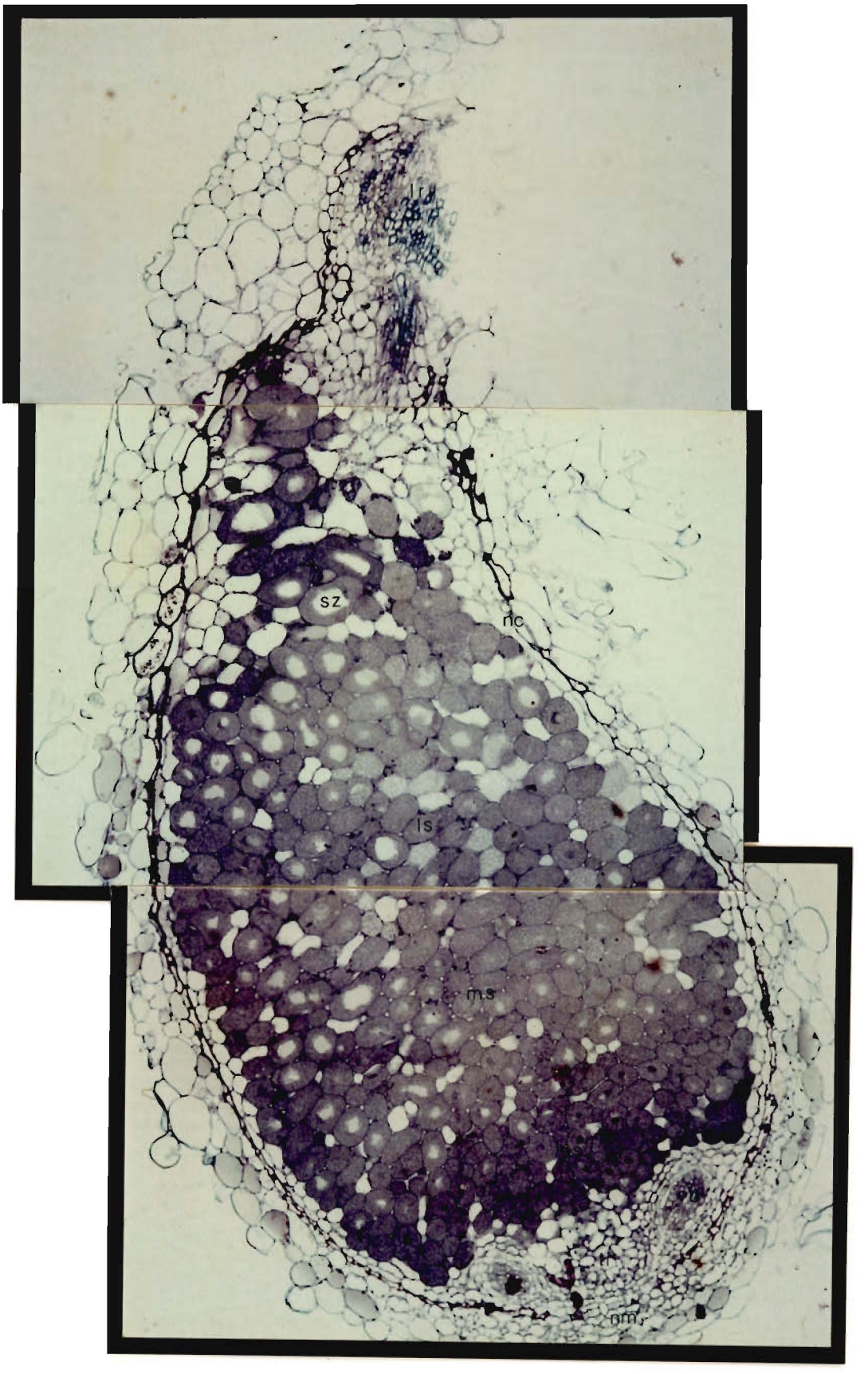
(a)Determinate nodules. These do not have persistent meristems. The vascular system becomes closed and there is usually little or no involvement of infection threads in the distribution of bacteria to the nodule cells.

(b)Indeterminate nodules. These have persistent meristems, resulting in a nodule all of whose tissues are of graded age from the growing point to the root attachment. Consequently the vascular system is open, due to juvenile cells near the meristem. Infection threads are the major mechanism for distribution of bacteria to the nodule cells (Bergersen 1982). *Trifolium repens* (white clover)/*Rhizobium trifolii* nodules are of this class. As this is the symbiosis on which this study focuses, only indeterminate nodules will be described here.

13.2.2. Infection.

The microsymbiont, a rhizobial species or strain specific to a certain

Colour Plate 3. Light micrograph montage of a median longitudinal section of a four week-old white clover (*Trifolium repens*) nodule stained with Toluidine blue. The main zones, as discussed in the text, are as follows; The nodule meristem (nm), the region of infection thread invasion (ti), the infected cells of the early (es), mature (ms) and late (ls) symbiotic phases, and the senescent zone (sz). The nodule is bounded by a mantle of nodule cortex (nc) and is attached to the lateral root (lr). Vascular bundles (vb) lie beneath the nodule cortex and pass around the central tissue to join at the base of the nodules. x 350.


sz
nc
is
m.s
nm

becomes attracted and attached to a root hair through which it subsequently infects the host plant. Root cortical tissue responds to rhizobial infection by developing polyploid cells and by rapid meristematic activity (Sutton 1983;Newcomb 1981). Rhizobia excrete auxins, cytokinins and other metabolites thought to be involved in initiating the DNA synthesis associated with endoreduplication and mitotic activity (Syono *et al.*1976).

13.3.2. The outer cortex.

All indeterminate nodules are invested in a spongy cortex divided into inner and outer layers by an endodermis open at the undifferentiated tip of the nodule (color plate 3). The outer cortex has large intercellular spaces. These cells have very little cytoplasm and are "gas-filled" (Bergersen 1982).

13.2.4. The endodermis.

The nodule endodermis is distinct and continuous from positions just proximal to the nodule meristem and fuses with the endodermis surrounding the root stele near the point of nodule attachment (color plate 3).

13.2.5. Interstitial cells.

Interstitial (uninfected) cells throughout the nodule tissue provide a large proportion of the interface with bacteriod-containing cells. These interfaces appear to be active sites of metabolic exchange with many symplastic connections to bacteriod containing cells (Bergersen 1983). It is believed they may also act as a route for gas exchange (Tu 1974b). Interstitial cells have prominent nuclei, large vacuoles and a normal complement of cytoplasmic organelles typical of parenchyma cells.

13.2.6. Vascular traces.

Beneath the nodule endodermis lie the vascular traces Plate 1.A). These pass around the central tissue to join at the base of the nodule and enter the vascular system of the root (color plate 3). Each vascular trace is contained by its own endodermis, within which lie pericycle cells surrounding xylem and phloem tissue. The pericycle is usually 1 to 5 cells thick. The cells have distinctive wall ingrowths that extend into all regions of the cell cytoplasm (small arrowheads). These cells are referred to as transfer cells (Pate *et al.*1969).

Symplastic connections can be traced from the sieve elements through the pericycle, vascular endodermis and cortex to the bacteriod filled cells of the central tissue, and constitute a pathway for the transfer of material between the host vascular system and the central tissue (Pate *et al.*1969).

13.2.7. The inner cortex.

The cells of the inner cortex directly beneath the endodermis are tightly packed. There are small but well-defined intercellular spaces. This layer is usually 2 to 4 cells deep. Nearer to the central tissue the cortical cells are thin-walled and indistinguishable in structure from the uninfected interstitial cells of the central tissue (color plate 3).

13.2.8. Bacteriod tissue.

All indeterminate nodules contain a central relatively large volume of tissue composed of enlarged ovoid parenchyma cells, whose cytoplasm is packed with symbiotic forms of rhizobia, the bacteriods (color plate 3). These central cells have a prominent central vacuole. The host cytoplasm occupies only a small proportion of the host cell volume. The host cytoplasmic components (cytoplasm, nucleus, amyloplasts and mitochondria) have been estimated to occupy only about 20% of the cell space, whilst the bacteriods account for 80% of the cell volume (Bergersen 1982;Goodchild 1977).

13.3. Ultrastructure and development of effective nodules.

13.3.1. Infection.

Normally uninfected root hairs are straight (Bauer 1981). Inoculation of legumes with either rhizobia or a culture filtrate produces deformation, either a curling or branching of the root hair (Dart 1974), generally only deformed root hairs become infected. An infection originates from the curl in the root hair (Calvert *et al.*1984).

13.3.1.1. Infection thread development.

The infection takes the form of a tubular thread within which the rhizobia are surrounded by a polysaccharide matrix (Plate 1.C)(Nutman 1948). Infection thread formation is not merely an invagination of the host cell wall but is an active process involving a localized host cell wall degradation and redirected cell wall growth (Quispel 1983;Goodchild 1977). The infection thread proceeds to grow towards the base of the root hair cell and may become branched. The host plasma membrane separates the host cytoplasm from the thread wall (Plate 1.E. - large arrowheads).

13.3.1.2. Bacteria in the infection thread.

Rhizobia in the infection thread are identical in structure to vegetative bacteria in the rhizosphere (Plate 1.D). The rhizobial cell wall consists of two bilayered membranes. The outer membrane, or cell envelope, consists of a thin outer layer and a more electron dense inner layer (Plate 1.E - small arrowheads). The inner, or cytoplasmic, membrane surrounding the bacterial cytoplasm is often found to be separated from the outer membrane, forming a periplasmic space (Sutton *et al.*1981).

The bacteria are often surrounded by a thin electron-opaque area, considered either a bacterial capsule or an artifact (Plate 1.C. - small arrowhead), and a finely granular moderately electron dense matrix composed of a mucopolysaccharide believed to be synthesized by the bacteria (Dart 1974)

13.3.1.3. Bacterial release.

The growing tip of the infection thread in clover root hairs is unwalled. Rhizobia are released from this and other unwalled regions of the infection thread by a process of endocytosis (Plate 1.B). Bacteria move towards an unwalled section of infection thread becoming closely associated with the surrounding host plasma membrane which forms a bulge around the rhizobia (Plate 1.D - arrowheads). The bulge becomes larger until the membrane pinches off so that the escaping rhizobium is surrounded by a peribacterial membrane which is initially derived from the host plasma membrane (Plate 2.A)(Tu 1974a;Dixon 1967;1969). Once the bacterium is released from the infection thread it is known as a bacteriod (Goodchild 1977). Poly-*B*-hydroxybutyric acid (PHB) is present in *R.trifolii* bacteria within infection threads (Plate 1.D - large arrowheads). This tends to be lost as the bacteria are released into the host cell cytoplasm (Mosse 1964).

In white clover each bacteriod is enclosed individually by a peri-bacteriod membrane which enlarges as the bacteriod volume increases. Coated and smooth vesicles are present in association with the unwalled region of the infection thread, and

Plate 1. Normal nodule tissue.

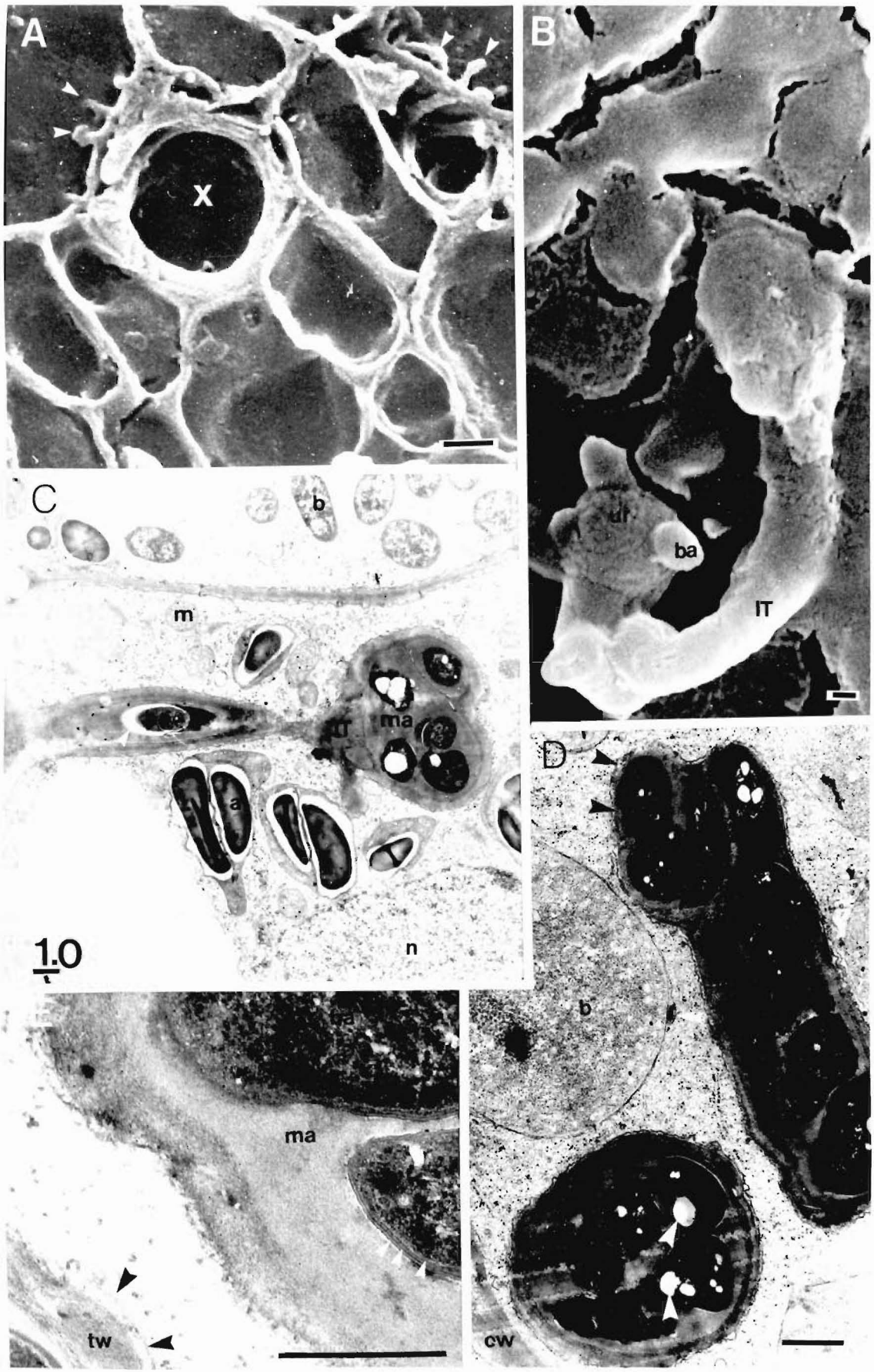
A. Nodule vascular trace, note pericycle cells containing many wall ingrowths (arrowed). x= xylem vessel.

B. Infection thread (it) within a normal nodule cell. Bacteria (ba) move toward an unwalled region (ur) of an infection thread and escape by endocytosis.

C. Bacteria (ba) within an infection thread (it) of an immature nodule cell. Bacteria are often surrounded by a thin electron-opaque area (arrowed), and a finely granular matrix material (ma). m=mitochondria, b=bacteriod, n=nucleus, a=amyloplast.

D. Bacteria in the infection thread often become densely packed, and contain poly-*B* hydroxybutyric acid (PHB) (white arrows). Bacteria move toward an unwalled section of infection thread which forms a bulge about the bacteria (black arrows). Released bacteriods often contain dense material in their peribacteriod space (double arrowhead).

E. Bacteria in the infection thread have identical morphology to free living rhizobia. The rhizobial cell wall consists of 2 bilayered membranes (white arrows). The host plasma membrane separates the host cytoplasm from the thread wall (black arrows). ba=bacteria, ma=matrix, tw=thread wall.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the enlarging peribacteriod membranes. It is thought that these vesicles supply material for growth of membranes and infection thread walls (Tu 1976;Robertson *et al.*1978a, 1978b;Robertson and Lyttelton 1982). An alternative hypothesis of vesicle function proposes that vesicles actively degrade the infection thread wall, creating the unwalled regions through which bacteria escape (Bassett *et al.*1977a).

A third possibility is that both the above processes operate simultaneously, degrading wall areas to allow bacterial escape from the infection thread, while growth of peribacterial membranes and maintenance of other infection thread regions continues. A nett growth system must be in operation as peribacteriod membranes expand in conjunction with the growing bacteriod. More biochemical evidence is required to confirm which hypothesis is correct.

13.3.2. The early symbiotic stage.

Indeterminate nodules have tissue of graded age. This review is therefore as much developmental as it is a description of the regions of a mature nodule.

13.3.2.1. Bacteriod differentiation.

Bacteriods at this stage are considered to be immature, divide (Plate 2.B - arrowhead and Plate 2.D. - arrowhead), lack nitrogenase activity and are present in nodule tissue that has not yet produced significant quantities of leghemoglobin (Sutton 1981).

The peribacterial membrane adheres to the outer surface of the bacterial membrane and divides with the bacteriod, keeping the ratio of bacteriod to peribacteriod membranes one to one (Robertson *et al.*1983) (Plate 2.A - arrowheads). Bacteriod walls are much thinner and less rigid than bacterial walls (Mackenzie *et al.*1973) and will be referred to here as the bacteriod outer membrane. This may facilitate passage of nutrients and fixed nitrogen between the bacteriod and the plant.

Bacterial ribosomes are concentrated at the bacteriod periphery, while the bacterial nuclear material becomes fibrous and dispersed (Sutton 1983). The number of bacteriods within the host cell increases markedly during the early symbiotic phase. Initially the bacteriods are restricted to the outer regions of the host cytoplasm but are eventually evenly distributed throughout.

13.3.2.2. Host cell differentiation.

Release of rhizobia from infection threads initiates, or at least coincides with, a number of changes in the host cell. The host cell endomembrane system becomes more extensive during the early symbiotic phase (Plate 2.A) with numerous golgi bodies and rough endoplasmic reticulum becoming prominent. An abundance of free ribosomes and polyribosomes results in a densely stained cytoplasm in young infected cells (Plate 2.C). There is a marked increase in host cell volume and an associated increase in the numbers of proplastids,amyloplasts and mitochondria. These organelles are usually located at the cell periphery near the cell wall (Plate 2.D), particularly near intercellular spaces or interstitial cells.

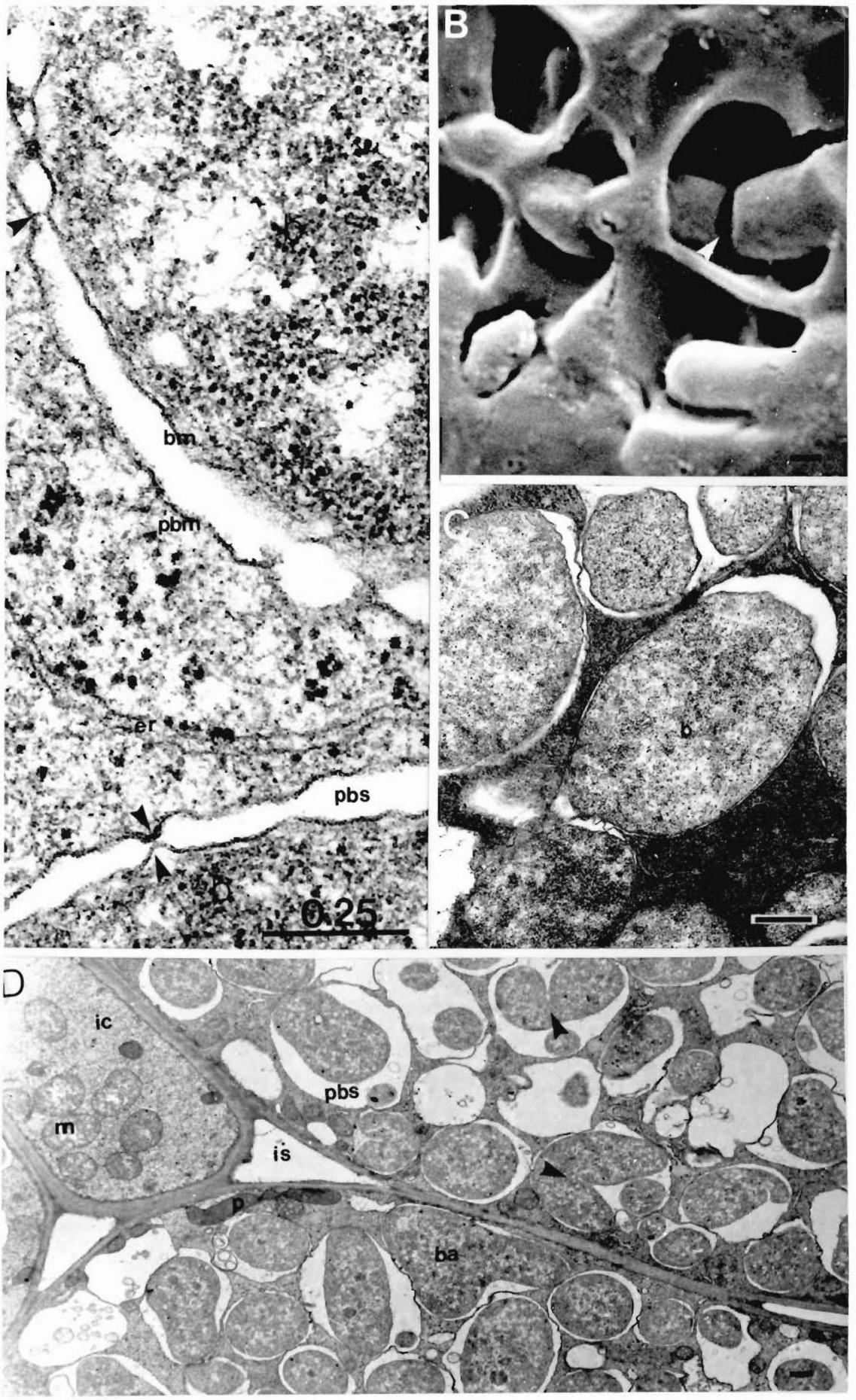
Plate 2. Normal nodule tissue.

A. The peribacteriod membrane (pbm) adheres to the outer surface of the bacterial membrane (bm) (arrows) across the peribacteriod space (pbs). The host cell endomembrane system becomes more extensive during the early symbiotic phase. er=endoplasmic reticulum, b=bacteriod.

B. Immature bacteriods divide (arrow) and separate into individual peribacteriod membranes.

C. An abundance of free ribosomes and polyribosomes results in a densely stained cytoplasm (dc). b=bacteriod.

D. Proplastids (p) and mitochondria (m) locate at the periphery of infected cells, particularly near intercellular spaces (is), but remain central in interstitial cells (ic). Immature bacteriods divide (arrows) and separate into individual peribacteriod membranes. ba=bacteriod, pbs=peribacteriod space.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It has been suggested that low oxygen concentrations are a key factor in normal bacteriod development. Low availability of free oxygen in the host is thought to result from high bacteriodal respiration rates, restricted diffusion of oxygen through intercellular spaces (Goodchild and Bergersen 1966) and the oxygen buffering and diffusion facilitating effects of the high leghemoglobin concentrations in infected cells (Sutton 1983). Leghemoglobin is present in the host cytosol from this stage (Melik Sarkisyan *et al.*1982;Robertson *et al.*1978b). Globin is a product of plant genes while bacteriods produce heam (Beringer *et al.*1979). Robertson *et al.*(1984) used Immunogol staining on thin sections of pea nodules to show leghemoglobin localized in the host cytoplasm.

Initially the starch grains of the amyloplasts are small and spherical (Plate 3.A). At the time when rhizobia reach their final density (early symbiotic phase) the starch grains become large and characteristically flattened (Mosse 1964) (Plate 3.B). The large central vacuole found during the early symbiotic phase presumably forms in part by coalescence of small vacuoles and by their subsequent growth. The nucleus increases in volume and DNA content.

13.3.3. The late symbiotic (mature) stage.

13.3.3.1. Bacteriod structure.

Late symbiotic development is characterized by changes in the size, morphology and cytology of the bacteriods which become enlarged and assume a spherical to polyhedral outline. The mature bacteriods do not divide and have been estimated to occupy a volume 40 times that of the free-living rhizobia (Gourret and Fernandez-Arias 1974). Mature bacteriods are characterized by a high nitrogenase activity and are found in tissue with a high leghemoglobin content. Nitrogenase activity was found to peak at 20 days after inoculation of *G.max* with *R.japonicum* but this period varies dependant on environmental factors (Bergersen 1982).

The bacteriod nucleoid remains dispersed and associated granules appear (Plate 3.C.). A plasma membrane derived vesicular system forms in the bacteriod cytoplasm with vesicles of approximately 0.1 micrometres in diameter (Plate 3.C. - arrowheads) (Gourret and Fernandez-Arias 1974). This has been described as a mesosome system (Dart and Mercer 1963). Tubules and sac-like shapes have also been observed in late symbiotic bacteria - these were attributed to fixation artifacts (Gourret and Fernandez-Arias 1974). Ribosomes become evenly dispersed throughout the bacteriod cytoplasm (Sutton 1983). Electron dense material is often observed in the peri-bacteriod space (Plate 1.D. - double arrowhead) (Gourret and Fernandez-Arias 1974). Polyphosphate granules have been observed associated with the nuclear material of bacteriods (van Brussel *et al.*1979) as have glycogen granules (Gourret and Fernandez-Arias 1974) which appear polyhedral.

In *Trifolium repens/R.trifolii* nodules the peri-bacteriod membrane is closely appressed to the bacteriod wall at maturity (Mosse 1964). This membrane is essential for nitrogen-fixation (Laane 1978;Melik-Sarkisyan *et al.*1982).

Plate 3. Normal nodule tissue.

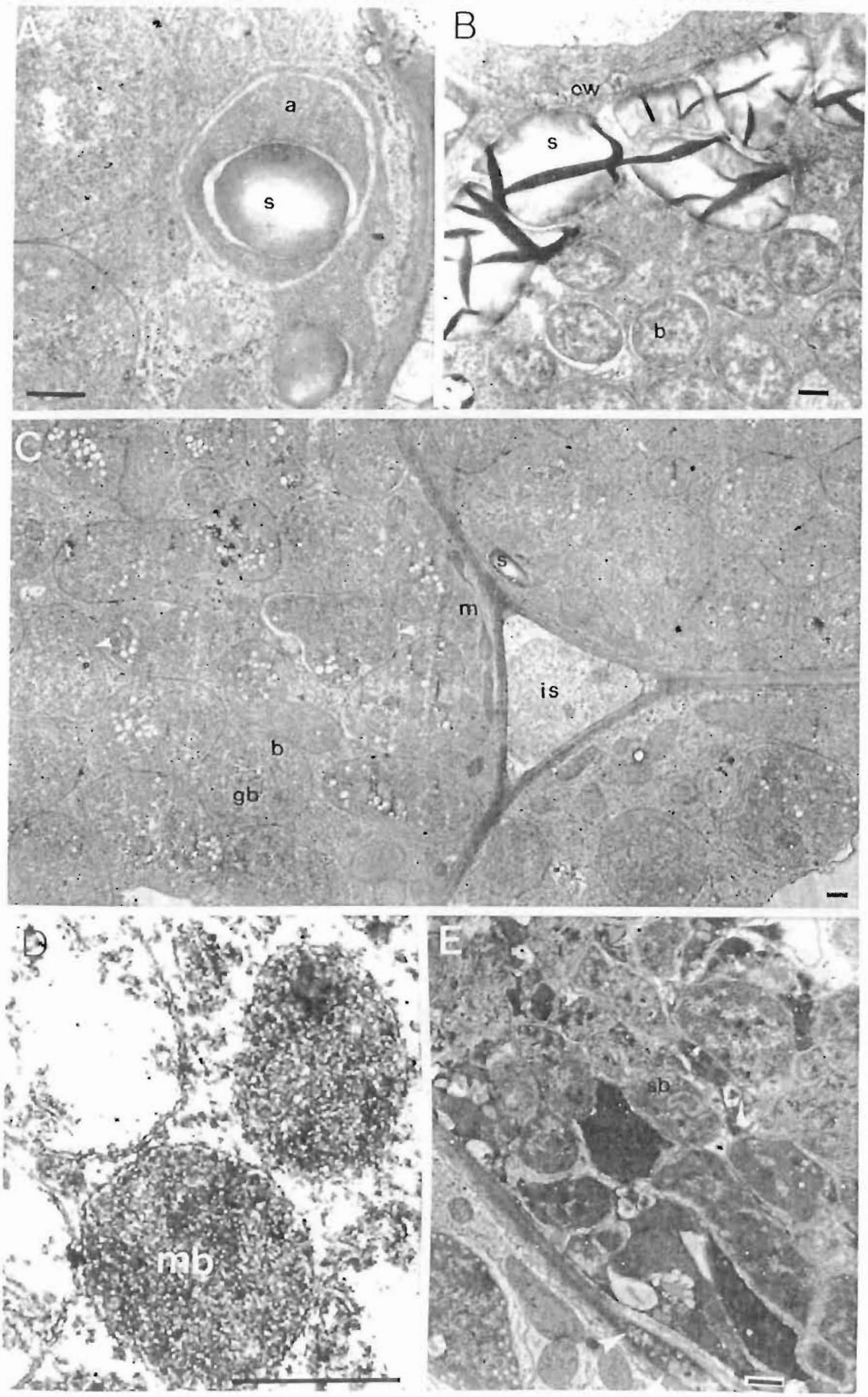
A. Amyloplasts (a) are located near the infected cell periphery and contain starch (s) that is small and spherical in immature tissue.

B. Mature cells contain starch (s) that is characteristically large and flattened. this starch lines the cell periphery. cw=cell wall, b=bacteriod.

C. Cells of mature tissue contain large numbers of bacteriods (b) which have characteristic granular bodies (gb). A plasma membrane-derived vesicular system forms (arrow). is=intercellular space, m=mitochondria, s=starch.

D. Microbodies (mb) are frequently observed in uninfected cells.

E. Senescent tissue contains senescing bacteriods (sb). Cell walls split along the middle lamella (arrows).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13.3.3.2. Host cell structure.

The host cell nucleus at maturity is greatly enlarged and amoeboid in shape and lies against the central vacuole. The host cytoplasm occupies only a small proportion of the host cell volume (Bergersen 1982). Ribosomes and rough endoplasmic reticulum are reduced in number. Similarly golgi bodies are less prominent in mature nodules (Goodchild and Bergersen 1966). Mitochondria and amyloplasts remain at the periphery of the host cell and concentrate near gas-filled intercellular spaces (Plate 3.C). At maturity the host cell cytoplasm becomes "featureless" (Goodchild and Bergersen 1966). Observations of microbodies in uninfected cells of *G.max* and *Phaseolus vulgaris* suggests that the final steps of biosynthesis of ureides allantoin and allantoic acid involves the microbodies and smooth E.R (Plate 3.D)(Newcomb 1981).

13.3.4. The senescent stage.

Senescent bacteriods represent the terminal stage of the nodule symbiosis, when nitrogenase activity and leghemoglobin content decline and peribacteriod membranes disintegrate (Sutton 1981) (Plate 3.E). Host cell cytoplasm disintegrates with associated deterioration of organelles. As senescence continues bacteriods form masses around the host cell nucleus. The host cell nucleus loses shape and structure. Multiple vacuoles appear in bacteriods, which then disintegrate (Sutton 1983) and vegetative bacteria proliferate in intercellular spaces.

The final stage of senescence is when the host cell wall breaks down,often by splitting at the middle lamella (Plate 3.E. - arrowhead). Young actively dividing vegetative bacteria proliferate in the cavities of the split wall and also amongst the collapsing bacteriods (Mosse 1964). These bacteria are believed to originate from remnants of the infection thread (Bergersen 1982). It has been suggested that senescing tissue may serve as a reservoir of infection from which new nodule growth arises (Bergersen 1982).

Chapter 14.0. Methods and Materials for Electron Microscopy.

14.1. Transmission electron microscopy.(T.E.M.)

Plants from experimental *in vitro* work were sampled for T.E.M. All nodules above 1mm in diameter were taken from each group of herbicide treated and control plants. Nodules were cut into two pieces, larger nodules were cut into slices no greater then 1mm thick.

The majority of the methods employed in the processing of the plant material followed standard procedures (see Hayat 1970,1972). Procedures used for processing material for T.E.M. work are shown in table 8. Unless otherwise stated all bar scales on micrographs represent 0.5 micrometres.

TABLE 8.Procedures for Transmission Electron Microscopy.

1.Primary Fixation. 3 hours in 3 % Glutaraldehyde (50 % Biological grade) in 0.025M Sörenson's phosphate buffer under vacuum.

2.Buffer Washes. After primary fixation pieces of tissue were washed in 0.025M Sörenson's phosphate buffer (pH 7-8) with 3 changes each of 20 minutes duration.

3.Post Fixation. 3 hours in 1% Osmium tetroxide in 0.025M phosphate buffer.

4.Post Fixation Buffering. Overnight in 0.025M Sörenson's phosphate buffer.

5.Dehydration in an ANALAR acetone series with 20 minutes each in 20,40,60 and 80 % acetone followed by two 30 minute changes in 100% acetone.

6.Infiltration. 5-6 hours in 25% Spurrs resin (from Hayat 1972) in 100% acetone followed by overnight in 50% Spurrs resin in 100% acetone.

7.Embedding in Spurrs (from Hayat 1972) low viscosity resin polymerized for 24-48 hours at 70^{o}C.

8.Ultramicrotomy. Specimens were cut out of resin blocks, glued to araldite stubs and hand trimmed to form blocks.

Blocks were surveyed by cutting 4 micron thick sections on an L.K.B. pyramitome (type 11800) equipped with glass knives. sections were stained with 0.5% toluidine blue in 1% sodium tetraborate (borax)(Newcomb 1976).

Ultrathin sections were cut on an L.K.B. Ultratome using glass knives. Sections were collected on uncoated 300 and 400 mesh, and coated 100 mesh copper grids.

9.Staining of thin sections. Grids were stained with Sato's (1967) lead citrate and Uranyl acetate (Watson 1958) solutions, see Hayat (1975).

10.Examination. All specimens were examined in a Hitachi HS-7S electron microscope at 50kV.

14.2. Light microscopy and photography.

Sections were cut at 1.5microns from blocks prepared for T.E.M. Sectioning was done using glass knives on a L.K.B. ultratome type 2128. Sections were stained with 0.55 toluidine blue in a 1% solution of sodium tetraborate (Borax) over a hot plate. Photomicrography was undertaken using a Leitz orthoplan microscope equipped with an orthomat photographic unit.

14.3. Scanning electron microscopy (S.E.M.).

S.E.M. examination of normal nodule tissue was carried out in order to further elucidate the structure of nodule cells. samples prepared for T.E.M. were modified for use in the S.E.M. by the method of Pring (1975). See Table 9 for procedure.

TABLE 9.Procedures for Scanning Electron Microscopy.

1.T.E.M. stubs to be examined in the S.E.M. were mounted in an ultramicrotome and faced off with a glass knife.

2.the smooth surface of the stub was etched with sodium ethoxide (2.5g sodium in 100ml dry ethanol). One drop of sodium ethoxide solution was allowed to drop on to the surface of the block every 5 seconds for 1 minute.

3.The etched block was rinsed once with a jet of ethanol, once with distilled water and finally once more with ethanol.

4.The block was allowed to air dry and was then affixed to a S.E.M. stub with Copper Print mountant.

5.Stubs were dessicated overnight and gold coated for several minutes in a Polaron diode Splutter device E5000.

6. Examination of specimens was conducted with a Cambridge Stereoscan 250 Mk 2 scanning electron microscope.

Chapter 15.0 Results of Effect of Herbicides on Ultrastructure of White Clover Nodules.

15.1. Effects of the herbicide paraquat on the ultrastructure of white clover nodules.

15.1.1. Early symbiotic tissue.

Early symbiotic bacteriod-containing cells exposed to paraquat are surrounded by many interstitial type cells (Plate 4.A). Tonoplasts in the interstitial cells are often pulled away from the cell cytoplasm leaving empty spaces (arrowhead). Cytoplasm of uninfected cells is dense and degenerate with damaged organelles. Early symbiotic infected cells exhibit an extremely dense host cell cytoplasm, and lack the usual layer of proplastids, amyloplasts and mitochondria along the host cell periphery. A few mitochondria present near intercellular spaces are severely damaged (Plate 4.A). Nuclei at this stage (Plate 4.B) are condensed.

Bacteriod cytoplasms are electron dense with fibrous areas, these probably being condensed nuclear material Plate 4.A). Bacteriod outer membranes are uneven and allow the bacteriods to assume unusual shapes (Plate 4.B. - arrow). Many peribacteriod membranes appear empty, others enclose a bacteriod but are discontinuous.

In some cells degeneration has proceeded further (Plate 4.C). Bacteriods show signs of recent division indicating that the tissue is still at the early symbiotic stage. Bacteriod cytoplasm is condensed, with peripheral clumping of cytoplasmic contents leaving central regions light and fibrous. Bacteriod cytoplasm have contracted in places leaving empty regions (Plate 4.C - arrow). Bacteriod outer membranes are highly uneven in profile and loose, often extending into the peribacteriod space (Plate 4.C. - small arrow).

Bacteriod cytoplasms remain condensed and bacteriods contort further (Plate 4.D). Peribacteriod membranes persist about the bacteriods, many of which have not separated following division resulting in two or more bacteriods per peribacteriod membrane. The bacteriods themselves are smaller than normal, being on average less than 1 micrometre in diameter, whereas normal bacteriods have diameters greater than 1 micrometre. Bacteriod outer membranes are uneven and detached in many places. There are many empty membrane-enclosed regions in the host cell cytoplasm which is granular and uneven. The bacteriod cytoplasmic matrix is apparently dispersed and ribosomes are aggregated. Some small vesicles are present in the host cell cytoplasm (Plate 4.D. - arrow), however no endoplasmic reticulum or golgi bodies are present.

In some cells the host cytoplasm has deteriorated further (Plate 4.E). Peribacteriod membranes are ruptured and collapsed followed by the bacteriod outer membranes allowing bacteriod cytoplasmic contents to spill into the surrounding host cell (Plate 4.E.).

Plate 4. Paraquat treated nodules.

A. Early symbiotic nodule tissue contain many empty interstitial-type cells (ic) whose plasmalemma is pulled away from the cell wall (arrows). Mitochondria (m) appear damaged. Bacteriods (b) are condensed.

B. Nuclei (n) of infected cells are condensed. Bacteriod (b) outer membranes are uneven (arrow).

C. Bacteriod (b) has recently divided but has not separated into individual peribacteriod membranes. The bacteriod cytoplasm is condensed and uneven, and has contracted away from the bacteriod membrane (arrows).

D. Bacteriod cytoplasms become very condensed and bacteriods assume unusual morphology. There are many empty peribacteriod membranes (pbm).

E. Peribacteriod membranes (pbm) have ruptured and collapsed, followed by the bacteriod outer membrane, allowing cytoplasmic contents to spill into the host cell (arrow).

F. Mature symbiotic bacteriods develop regions of high electron density at their periphery (large arrows). Bacteriod membranes are diffuse (small arrows).gb=granular body.

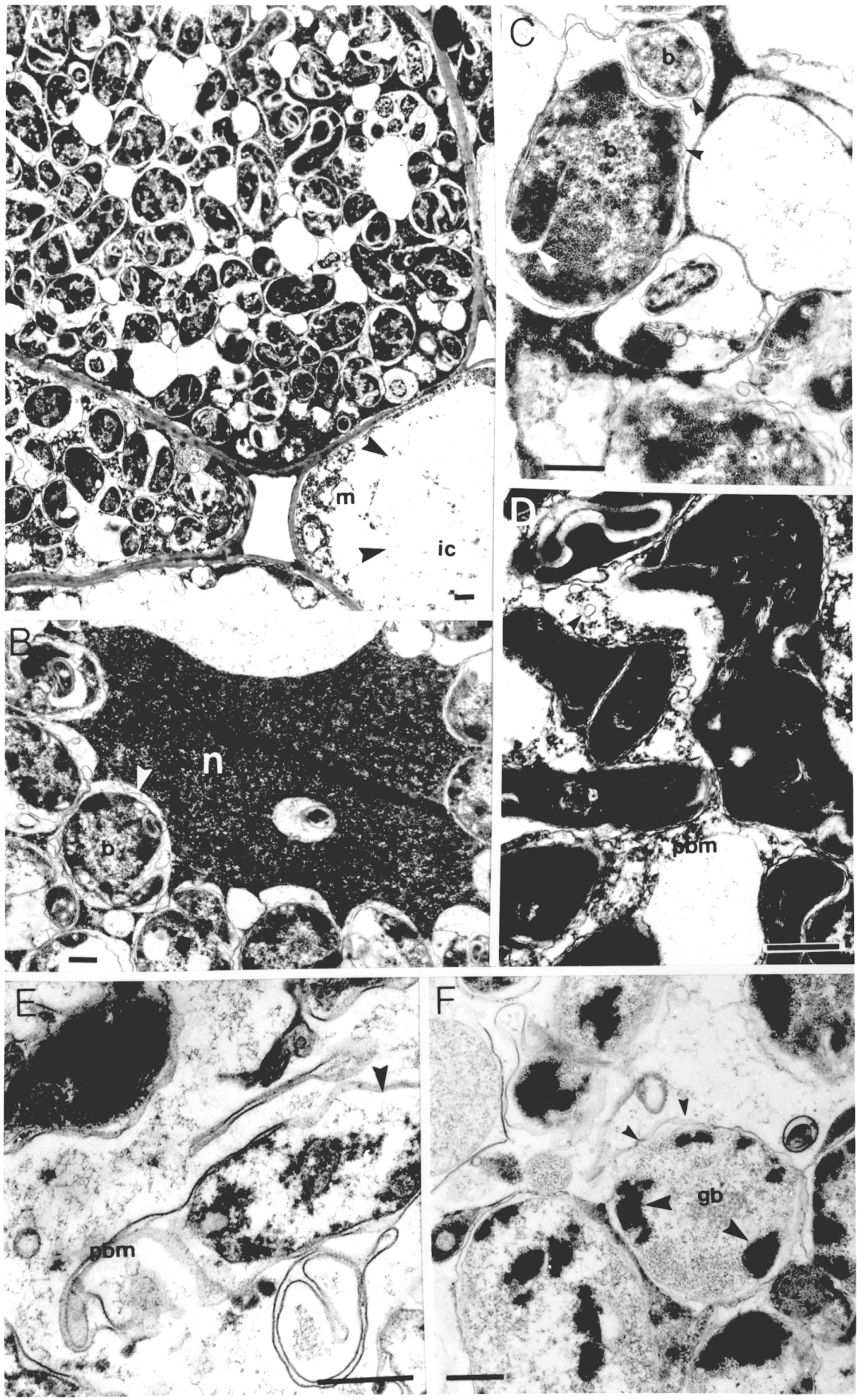
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Plate 5. Paraquat treated nodules.

A. Bacteriod outer membranes detach from the bacteriod cytoplasm (large arrows) and appear to fuse. gb=granular body.

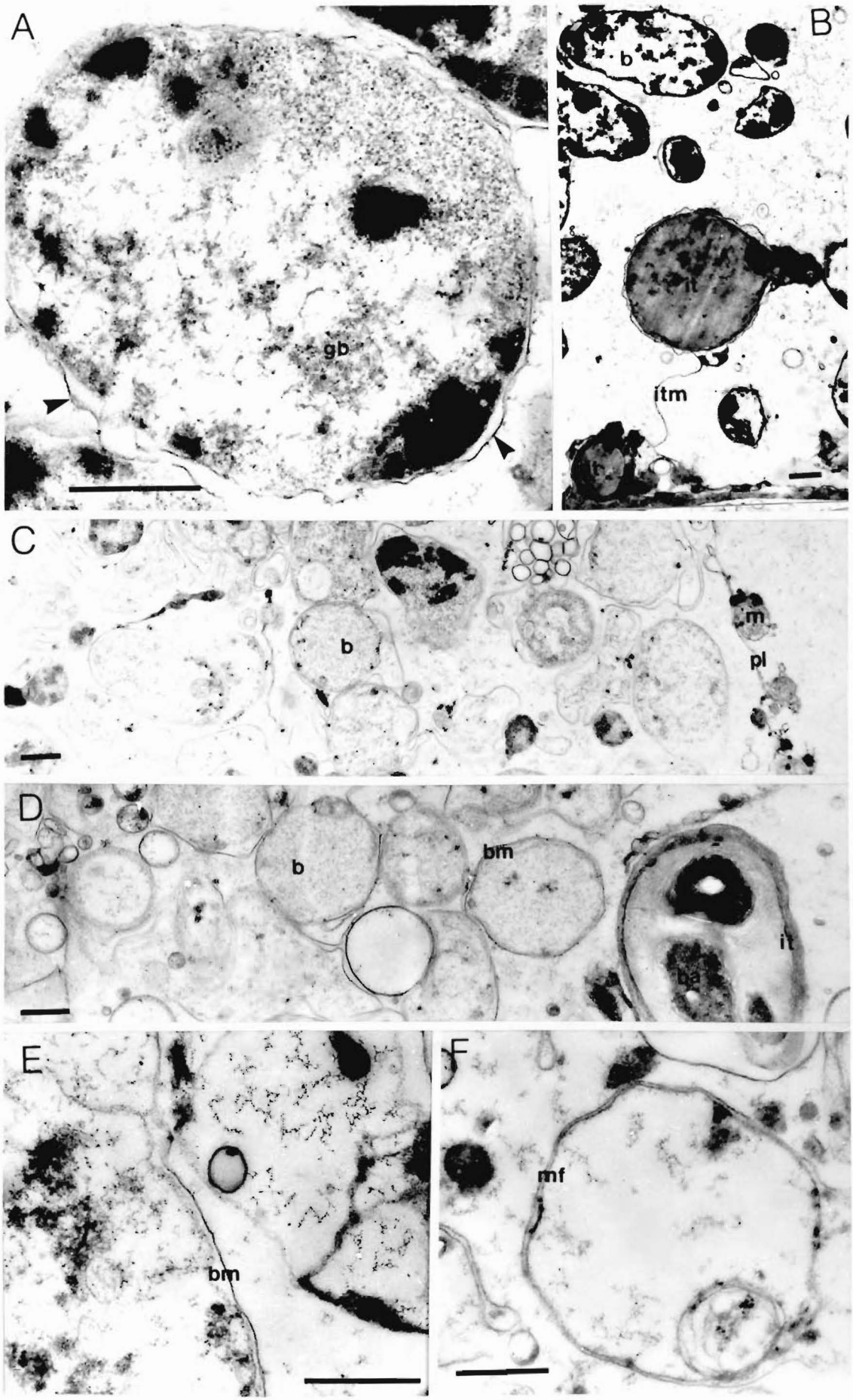
B. Bacteriods (b) swell and contain electron dense peripheral regions. Infection threads are present (it), and the infection thread membrane is intact.

C. The plasmalemma (pl) detachs from the host cell wall and encloses the mass of degenerate bacteriods (b). m=mitochondria.

D. Infection threads (it) containing normal bacteria (ba) are present in cells full of degenerate bacteriods (b). Bacteriod membranes (bm) contain little bacteriod cytoplasm.

E. Bacteriod cytoplasmic material escapes into the host cell cytoplasm through the degenerate bacteriod membrane (bm).

F. Membrane fragments (mf) fuse within the degenerate cell.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15.1.2. Mature symbiotic tissue.

Mature symbiotic tissue exhibits a different pattern of degeneration. Bacteriods do not develop the extremely dense cytoplasm seen in early symbiotic tissue, instead regions of high electron density develop along their peripheries (Plate 4.F. - large arrows). These dense regions appear to be the result of an aggregation of bacteriod cytoplasmic material. Some bacteriods still contain granular bodies, but few exhibit any remains of peribacteriod membranes. Bacteriod outer membranes are intact but show signs of apparent imminent deterioration being uneven and diffuse in places (Plate 4.F. - small arrows).

The host cell cytoplasm has deteriorated leaving only fibrous material in the host cell. Plant cell walls remain intact but appear diffuse, indicating the possible onset of deterioration. Bacteriod outer membranes become detached from the bacteriod cytoplasm (Plate 5.A. - large arrows) and appear to be capable of fusing. The bacteriod granular bodies also appear to be dispersing.

The deteriorating mature bacteriods swell (Plate 5.B). Electron dense peripheral regions become more condensed leaving the remainder of the bacteriod cytoplasm empty, apart from some small clumps of ribosomes. Bacteriod membranes rupture and bacteriod cytoplasmic contents are released into the empty host cell. Infection threads are present and the plasmalemma of the host cell is also still intact but becomes detached from the host cell wall (Plate 5.C) and encloses the mass of deteriorating bacteriods. Occasional damaged mitochondria can be seen associated with this surrounding membrane.

Infection threads are prominent in the deteriorating cells (Plate 5.D). These may act as a source of vegetative bacteria at a later stage of deterioration. Occasional electron dense regions are still present in the bacteriod cytoplasm, but these are much smaller than those previously observed. Little of the bacteriod cytoplasm remains enclosed by the bacteriod membranes. The remains of the bacteriod cytoplasm disperse into the host cell (Plate 5.E), and bacteriod membranes also become dispersed. Eventually only wall and membrane fragments remain (Plate 5.F) which appear to fuse, forming tubules and vesicles in the empty host cell.

15.2. Effects of the herbicide MCPB on the ultrastructure of white clover nodules.

15.2.1. Early symbiotic tissue.

Some areas of early symbiotic tissue appear generally unaffected by growth in the presence of MCPB (Plate 6.A), however ruptured peribacteriod membranes are a frequent occurrence. These ruptures in the membrane may allow host cytoplasmic material to penetrate the peribacteriod space, fibrous material in the peribacteriod space suggests that this may have occurred. Alternatively this material may be bacteriod cytoplasmic contents lost through damaged bacteriod membranes. Bacteriods collapse due to infolding of the bacteriod outer membrane, which become diffuse in appearance.

In the early symbiotic stage groups of bacteriods often deteriorate simultaneously (Plate 6.B). These bacteriods are much smaller than the surrounding unaffected bacteriods seemingly due to a lack of expansion following release from the infection thread. These bacteriods lack contact with their peribacteriod membrane and collapse within their peribacteriod spaces.

Following condensation of the bacteriod cytoplasm the bacteriod membranes rupture, releasing cytoplasmic material into the peribacteriod space. Small vesicles are sometimes found in the peribacteriod space at this stage. The peribacteriod membrane also collapses after bacteriod degeneration. Infection threads and the emergence of bacteria do not appear to be affected by the herbicide treatment (Plate 6.C).

15.2.2. Mature symbiotic tissue.

Mature tissue also has a normal structure in some cells of the nodules of MCPB treated plants. These areas have normal mature bacteriods with granular bodies and intracytoplasmic vesicles. Other regions show considerable signs of damage. An early indication of negative effects of the herbicide treatment is the loss of contact between the bacteriod cytoplasm and outer membrane. The outer membrane often remains in contact with the peribacteriod membrane (Plate 6.D - arrows). The bacteriod cytoplasm is slightly more dense then normal, although the cytoplasm of the host cell appears unaffected.

Bacteriod cytoplasms condense (Plate 6.E). Many contain granular bodies but do not exhibit the intracytoplasmic vesicle system normally found in mature bacteriods. Extensive electron transparent regions appear in the bacteriod cytoplasm (Plate 6.E. - arrow). The bacteriod outer membrane loses structure and further detachs from the bacteriod cytoplasm. Peribacteriod membranes also lose structure and become detached, pulling away from the bacteriod. Vesicles are sometimes seen in the spaces formed by this loss of structure, possibly having entered through ruptures in the peribacteriod membrane (Plate 6.E. - double arrowhead).

The bacteriod cytoplasm develops electron dense areas near the periphery of the cell (Plate 6.F. - arrow). These dense regions appear to be the result of

Plate 6. MCPB treated nodules.

A. Some regions of early symbiotic tissue is little affected by the herbicide treatment. gb=granular body, b=bacteriod, cw=cell wall. Mitochondria (m) show damaged cristae.

B. Bacteriods (b) degenerate in groups. pbs=peribacteriod space.

C. Bacteria emerge normally from infection threads. ba=bacteria, PHB=poly-*B*-hydroxybutyric acid.

D. Bacteriods (b) lose contact between their outer membrane and the bacteriod cytoplasm (arrows). Mitochondria (m) have damaged cristae.

E. Damaged bacteriods (b) have very condensed cytoplasms, but still contain granular bodies (gb). Cytoplasms invaginate (white arrow) and vesicles associated with this are often present (double arrow).

F. The bacteriod cytoplasm develops electron dense regions within the cytoplasm (arrows). Bacteriods become extremely condensed (double arrowhead).

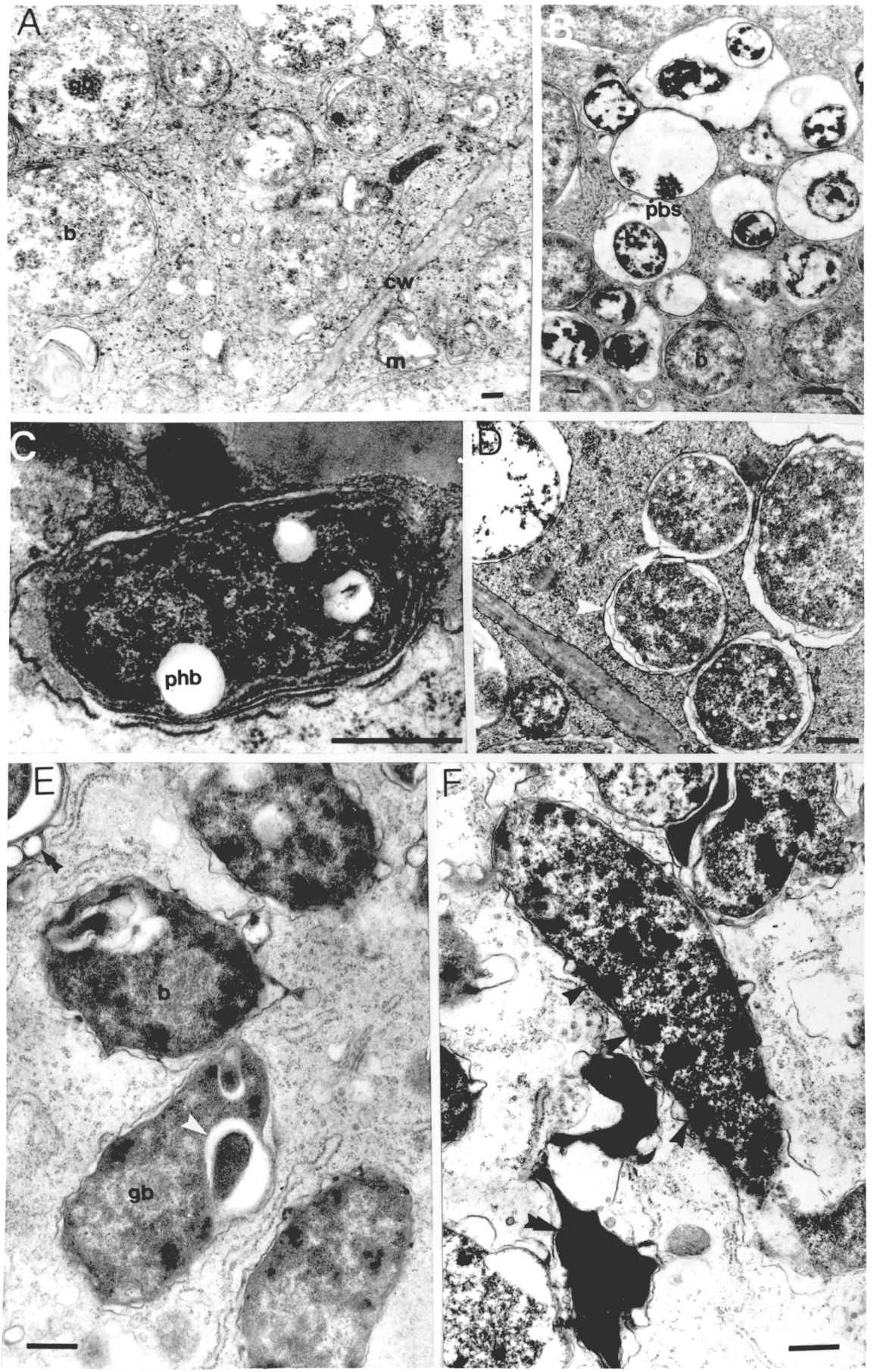
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Plate 7. MCPB treated nodules.

A. Vesicles are found in the peribacteriod space of bacteriods (b) (arrows). gb=granular body.

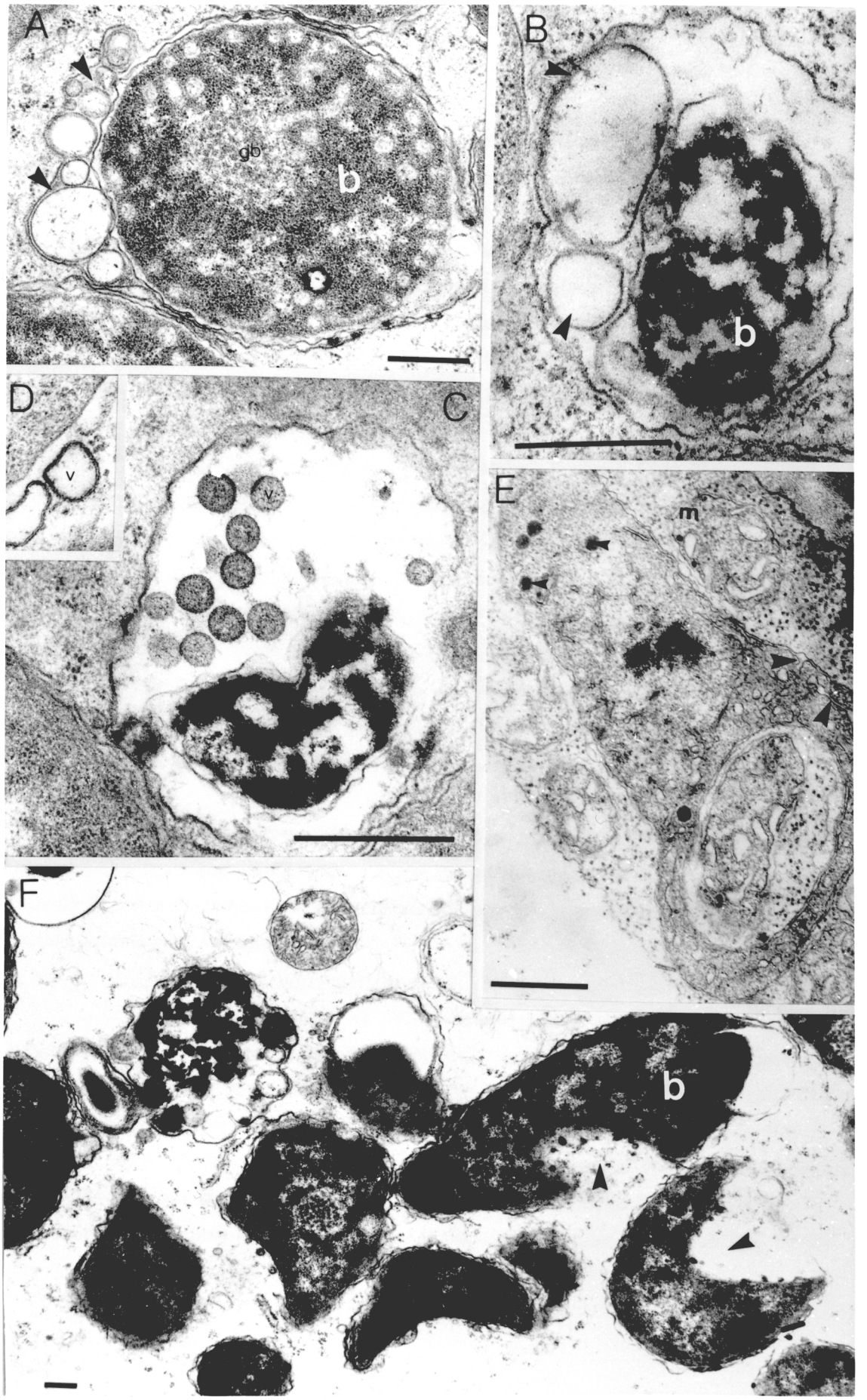
B. Vesicles in the peribacteriod space of bacteriods are often associated with degeneration of the bacteriod (b) (arrows).

C. Double membrane bound vesicles (v) containing a dense granular material are within a peribacteriod space contained by a damaged peribacteriod membrane.

D. Double membranes of vesicles (v) are clearly visible.

E. Proplastids often form invaginations containing mitochondria (m) with enlarged cristae. The proplastids contain osmiophilic globules (small arrows) and ferritin (f). Their inner membrane is highly infolded, forming tubules (large arrows).

F. Bacteriods (b) release cytoplasmic material into the host cell. Vesicles are associated with this localized deterioration, causing the bacteriods to become sickle-shaped in profile (arrows).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C
v
E
m
F
b

aggregation of cytoplasmic contents possibly due to disruption of the bacteriod cytoplasmic matrix. The remainder of the bacteriod cytoplasm exhibits a very uneven granular appearance. Peribacteriod membranes remain attached to the surface of the degenerating bacteriods.

Bacteriod cytoplasmic material is released into the host cell cytoplasm from areas of bacteriod membrane rupture (Plate 7.F - arrowed). Many small electron dense vesicles appear to be associated with this deterioration. Bacteriods take on a sickle shape in section due to the localized nature of this degradation.

The host cell cytoplasm degenerates. Membrane fragments, few scattered ribosomes and fragmented endoplasmic reticulum remain in the otherwise empty cell. Vesiculated rough endoplasmic reticulum is present. Bacteriods in extreme states of decay appear as electron dense bodies often still enclosed by fragments of membranes (Plate 6.F - double arrowhead).

15.2.3. Vesicles.

Unusually large numbers of vesicles are present in the cytoplasm of symbiotic nodule cells. These vesicles range in diameter from 74 to 520 nm. Several types of vesicles are associated with deterioration of bacteriods in nodules of MCPB treated plants. Double membrane bound vesicles in the size range 150-170nm in diameter are present. These are often associated with breaks in the peribacteriod membrane (Plate 7.C. and Plate 7.D.) or in the peribacteriod space of deteriorating bacteriods. When in peribacteriod spaces these vesicles appear to contain some material of moderate electron density, whereas those free in the cytoplasm are electron translucent.

A third commonly observed type of vesicle (Plate 7.AS. and 7.B.) ranges in size from 200 to 500nm in diameter. These are usually situated in the peribacteriod space or in the host cell cytoplasm, are bound by a single unit membrane and have electron transparent contents.

15.2.4. Host cell organelles.

Proplastids in nodules of MCPB treated plants often form invaginations.(Plate 7.E. - arrow). The proplastid inner membrane is highly infolded, forming numerous tubules and vesicles. Ferritin aggregates and osmiophilic globules of approximately 60nm diameter (Plate 7.E. - small arrows) are often present in the plastids of early and late symbiotic tissue. Mitochondria contain few cristae which are often enlarged, they also contain osmiophilic bodies of 30-35nm diameter. Mitochondria in mature tissue show frequent damage to cristae (Plate 6.A and Plate 6.D).

15.3. Effects of the herbicide Bentazone on the ultrastructure of white clover Nodules.

15.3.1. Preamble.

Bentazone is absorbed through the roots of plants and translocated. Soil application is more toxic than foliar applications as very little absorption occurs through the leaves (Skuterad and Caseley 1980). Therefore nodules are likely to be a target of bentazone activity.

15.3.2. Early symbiotic tissue.

Early symbiotic tissue in nodules on plants exposed to bentazone contain very expanded, irregular peribacteriod membranes, creating large peribacteriod spaces which contain fibrous material (Plate 8.B - arrow). Large vesicles are occasionally observed in these large peribacteriod spaces (Plate 8.B. - double arrow).

Bacteriod size and shape are highly variable, and their cytoplasms are of varying density (Plate 8.B). Bacteriod outer membranes and peribacteriod membranes are diffuse and may be broken in places allowing host cell or bacteriod cytoplasmic material into the peribacteriod space. Some empty peribacteriod membranes are present in the host cell cytoplasm, these deteriorate rapidly. Host cell cytoplasm is normal in appearance at this stage, being moderately dense, and containing frequent endoplasmic reticulum and numerous ribosomes.

15.3.2. Mature symbiotic tissue.

Mature bacteriods contain only small granular bodies (Plate 8.D) and have large numbers of intracytoplasmic vesicles (Plate 8.D. - arrow) which are very extensive, large and often ramifying into the centre of the bacteriods. These vesicles also appear to contain fibrous material. Bacteriods show signs of division without separation (Plate 8.D. - double arrow), causing 3 or more bacteriods to be contained within one peribacteriod membrane. Peribacteriod membranes show occasional small discontinuities (Plate 8.D. - small arrow). The host cell cytoplasm has little endoplasmic reticulum, golgi bodies or associated vesiculation and few ribosomes. Chloroplast-like plastids in interstitial cells are swollen and completely round, these have disorganized grana, and contain many osmiophilic plastoglobuli.

Deteriorating bacteriods in mature symbiotic cells contain small electron dense regions at their peripheries (Plate 8.C. - small arrow). The bacteriod cytoplasm is light and dispersed. Peribacteriod membranes are ruptured and fragmented, but they remain attached to the bacteriod (Plate 8.C. - small double arrow). Bacteriod outer membranes are intact but uneven in some cases. The host cell cytoplasm is dispersed with no organelles visible. Infection threads are present at this stage of deterioration (Plate 8.A) and appear undamaged even though tissue surrounding the infection thread is deteriorating (Plate 8.A. - arrow).

Bacteriod outer membranes rupture (Plate 9.A. - Large arrowhead) and release cytoplasmic material into the empty host cell cytoplasm. Many bacteriods still contain electron dense regions of clumped cytoplasmic material. Bacteriodal remains

Plate 8. Bentazone treated nodules.

A. Infection threads (it) remain intact in otherwise damaged cells. However bacteria in the infection thread (arrows) contain round inclusions.

B. Early symbiotic bacteriods (b) vary in density and have large irregular peribacteriod spaces containing fibrous material (arrow). Large vesicles are occasionally present in the peribacteriod space (double arrow).

C. Deteriorating bacteriods contain electron dense regions (single arrow). Peribacteriod membranes are uneven and detached from bacteriods (double arrows).

D. Mature bacteriods have large numbers of intracytoplasmic vesicles (white arrows). Bacteriods show signs of division without separation (double arrow) resulting in more than two bacteriods per peribacteriod membrane. Peribacteriod membranes are broken in places (small arrow).

E. Bacteriod remains appear as regions of fibrous material. Infection threads remain (arrows) although the infection thread walls are deteriorating. Bacteria (ba) remain enclosed by the infection thread wall, those released, deteriorate (double arrow). m=mitochondria.

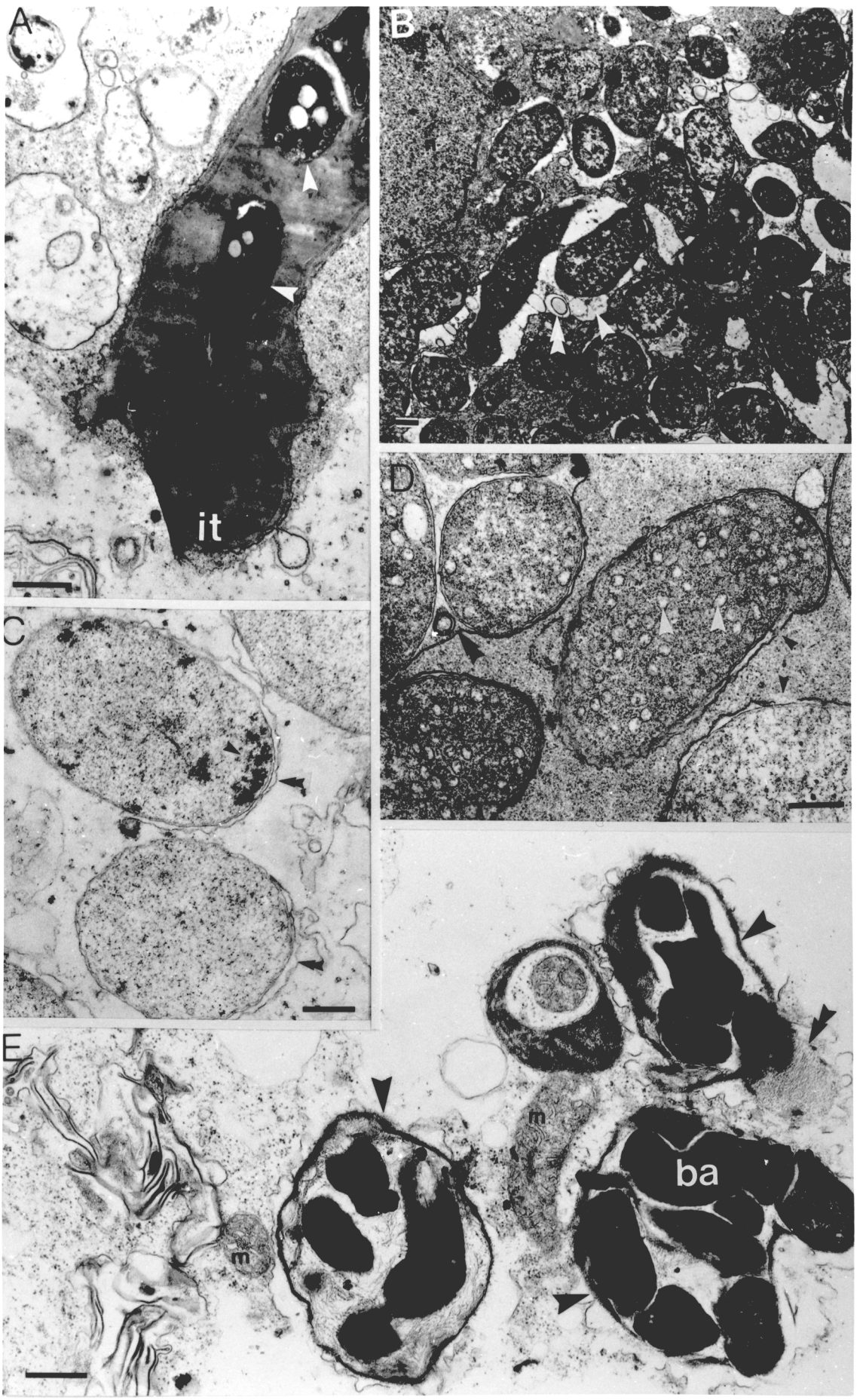

A
it
B
C
D
E
m
m
ba

Plate 9. Bentazone treated nodules.

A. Bacteriods (b) have ruptured outer membranes (arrow) and light fibrous cytoplasms.

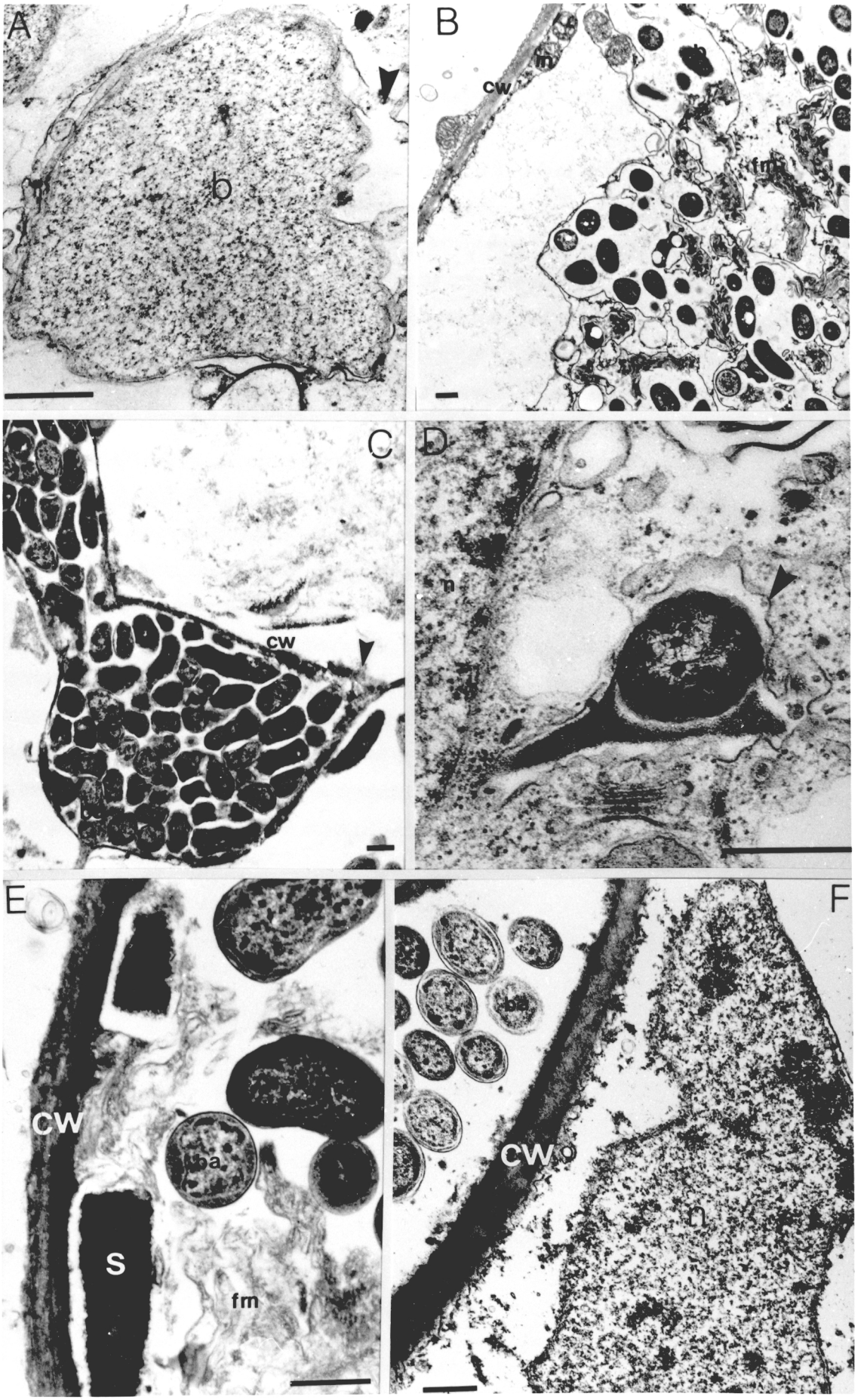
B. Large populations of bacteria (b) of vegetative structure are present among the fibrous remains (fm) of the cell cytoplasmic contents. cw=cell wall, m=mitochondria.

C. Host cells become packed with rhizobial bacteria (ba), and host cell walls rupture (arrow).

D. Budding dictyosomes (d) are present in degenerate host cells, which often contain an intact nucleus (n). A membrane surrounding a degenerate infection thread and bacteria, remains intact (arrow).

E. Starch (s) released from amyloplasts is found degenerating within the host cell. Vegetative bacteria (ba) thrive amongst the host cell remains (fm).

F. Nuclei (n) eventually detach from the cell wall (cw) of interstitial cells at late stages of breakdown. ba=bacteria.


A
b
B
cw
fm
C
cw
D
n
E
cw
ba
S
fm
F
cw
n

appear as regions of fibrous material (Plate 8.E), cytoplasmic contents having been released into the host cell.

15.3.4. Infection threads.

Deteriorated infection threads are present in degenerate cells (Plate 8.E. - large arrowhead). A membrane still surrounds the infection thread bacteria although the infection thread wall has dispersed. Bacteria in these infection threads lack PHB. Bacteria are being released from the infection thread due to the degenerate state of the infection thread wall, some of these bacteria are intact although others appear to be breaking down on contact with the host cell cytoplasm (Plate 8.E. - small double arrowhead). The bacteria released in this way may form the basis of the large population of vegetative bacteria that are present in many degenerate cells (Plate 9.B). These rhizobia flourish in the empty host cells and are identical to free living rhizobia. Cells become completely packed with rhizobia (Plate 9.C) and the host cell walls deteriorate (Plate 9.C. - arrow).

15.3.5. Host cell oprganelles.

Mitochondria are present along the host cell wall and among aggregated cell remains about the nucleus at very late stages of degeneration. Budding dictyosomes are also intact at very late stages of degeneration (Plate 9.D). The host cell nucleus remains intact (Plate 9.D) and bacteriod and host cell remains clump about it. Starch released from amyloplasts deteriorates in the host cell (Plate 9.E) among the saprophytic bacteria. Host nuclei of interstitial cells are torn from their position against the host cell wall (Plate 9.F) as the cytoplasm of uninfected cells also breaks down. The nucleus appears senescent with chromatin contracted into electron dense particles. The nuclear membrane ruptures only after most of the cell has deteriorated.

15.4. Effects of the herbicide Fusilade on ultrastructure of white clover nodules.

15.4.1. Early symbiotic tissue.

Bacteriods at the early symbiotic stage of development in nodules of plants grown in the presence of fusilade are often in groups within a single peribacteriod membrane (Plate 10.A). The bacteriods fail to separate into individual peribacteriod membranes following division. These bacteriods often do not maintain contact between their outer membranes and the peribacteriod membrane. The host cell cytoplasm is evenly dense and normal in appearance with large populations of vesicles, often containing fibrillar material. Large coated vesicles, or enlarged RER is also present (Plate 10.A. - arrow).

Bacteriods which have divided but not separated, deteriorate within their enlarged peribacteriod membrane.(Plate 10.B) These bacteriods often have not enlarged and some still contain PHB (Plate 10.B. - arrow) which is normally lost during bacteriod emergence from the infection thread. Bacteriod cytoplasm is condensed. Bacteriod outer membranes rupture and the bacteriod cytoplasm loses material into the peribacteriod space (Plate 10.B. - double arrow). The peribacteriod membranes remain intact. The host cell cytoplasm becomes extremely electron dense (Plate 10.B. - double white arrow). Bacteriods which would appear to have a normal structure may also deteriorate. These bacteriods also lack peribacteriod membrane-bacteriod outer membrane contacts (Plate 10.E) and lyse, releasing cytoplasmic material into the peribacteriod space (Plate 10.E. - small arrows). Vesicles are often associated with the later stages of these early degenerations and may be removing bacteriod remains, as little of this material is present in the peribacteriod space.

Intact bacteriods approaching maturity in nodules of plants exposed to fusilade, often have electron dense regions along their peripheries (Plate 10.C. - arrows). These regions appear granular and may be a result of clumped ribosomes. Electron transparent regions appear in bacteriod cytoplasms (Plate 10.D. - double arrowheads) often between the bacteriod cytoplasm and the cytoplasmic membrane. Bacteriod outer membranes alter shape and detach from the bacteriod cytoplasm in some instances (Plate 10.C. - double arrowheads). Mitochondria and pro-plastids are present against the host cell wall, but are extremely dense and difficult to identify.

Cells at a later stage of deterioration are also present (Plate 10.D). Large transparent regions become obvious in many of the bacteriods, whose outer membranes are uneven, in some instances appearing discontinuous and dispersed. Fibrous material is present in peribacteriod spaces (Plate 10.D. - arrows), which appears to be bacteriod cytoplasmic contents that have been released. Many electron dense regions are present in bacteriod cytoplasms (Plate 10.D. - small white arrows), is light and dispersed.

Plate 10. Fusilade treated nodules.

A. Bacteriods (b) divide but do not separate into individual peribacteriod membranes (pbm). Large coated vesicles (or possibly enlarged endoplasmic reticulum) are present in the host cell (arrow).

B. Peribacteriod membranes (pbm) contain many degenerate bacteriods, some of which appear to contain poly-*B*-hydroxybutyric acid (PHB). Bacteriod outer membranes are ruptured (double black arrow). The host cell cytoplasm is electron dense (double white arrow).

C. Electron dense regions (arrows) are present along the periphery of bacteriods. Bacteriod cytoplasms condense and pull away from the bacteriod membrane (double arrowhead). gb=granular body.

D. Large electron transparent regions appear in bacteriod cytoplasms (double arrowheads). Fibrous material is present in the peribacteriod spaces (single black arrows). Many electron dense regions are present in the bacteriod cytoplasms (small white arrows).

E. Degenerate bacteriods (b) lose contact with the peribacteriod membrane (pbm) and lyse (arrows). Vesicles (v) are associated with this deterioration. Mitochondria (m) are damaged. bm=bacteriod membrane.

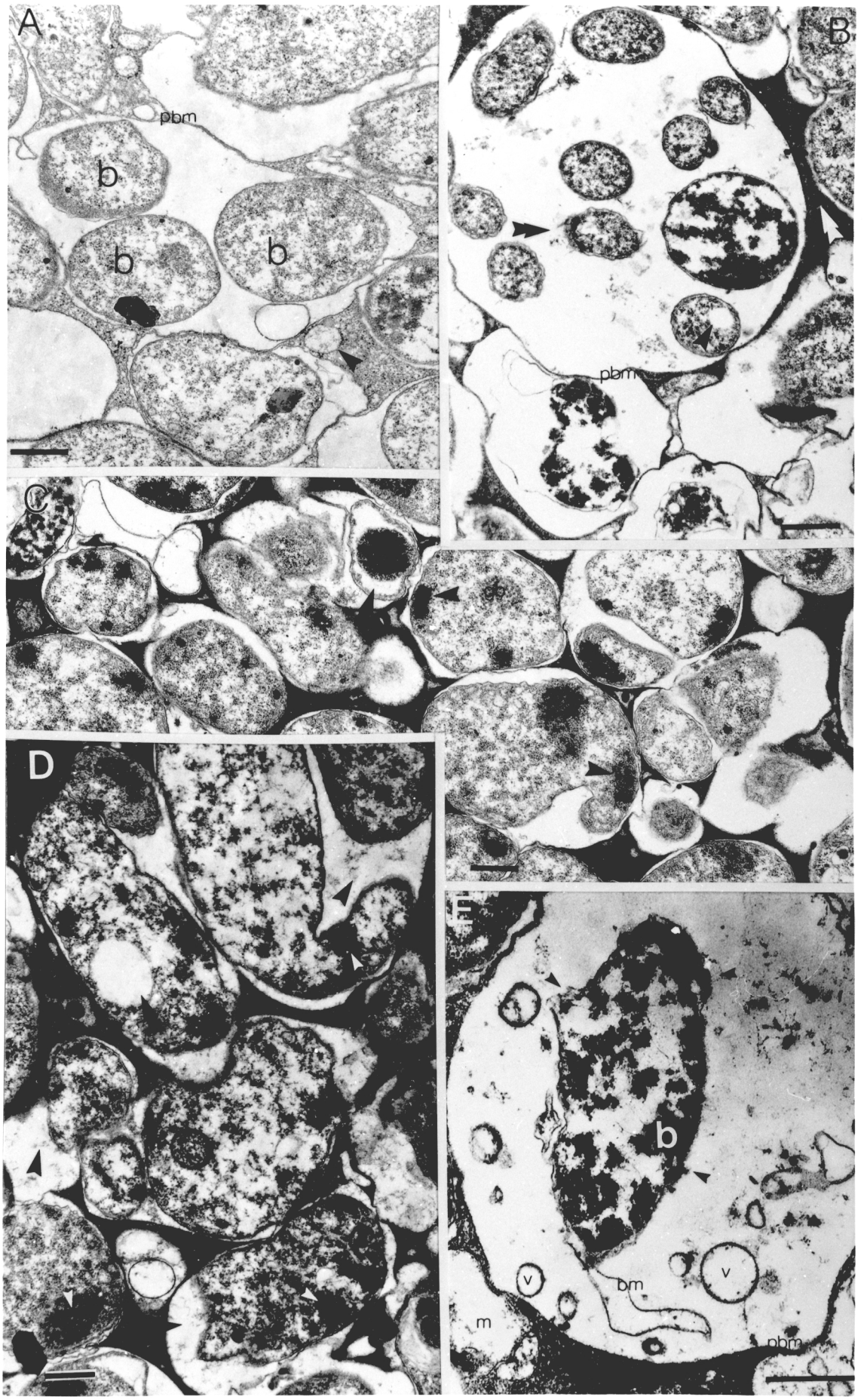

A
pbm
b
b
b
B
pbm
C
D
E
b
v
v
bm
m
pbm

Plate 11. Fusilade treated nodules.

A. Bacteriod (b) cytoplasms develop large electron dense regions at their periphery (large arrows). Peribacteriod membranes (pbm) are discontinuous (double arrowheads). Vesicles appear to be fusing to peribacteriod membranes (small arrows).

B. Degenerate bacteriods (b) occur in the same cell as bacteriods normal in appearance. Intracytoplasmic vesicles are enlarged (arrows). Some bacteriods appear to have a shrunken cytoplasm (double arrows).

C. Peribacteriod membranes become dispersed (small arrows). Electron dense regions are still present in the bacteriod cytoplasms (large arrow).

D. Bacteriod membranes rupture and release bacteriod cytoplasmic material (arrows).

E. Infection threads (it) remain intact and contain large numbers of bacteria (ba), but their surrounding membrane degenerates, although remaining attached to the infection thread (arrows).

F. The infection thread wall (itw) degenerated at the same time as the host cell wall (cw) (arrows), indicating they are of similar composition. itm=infection thread matrix, m=mitochondria.

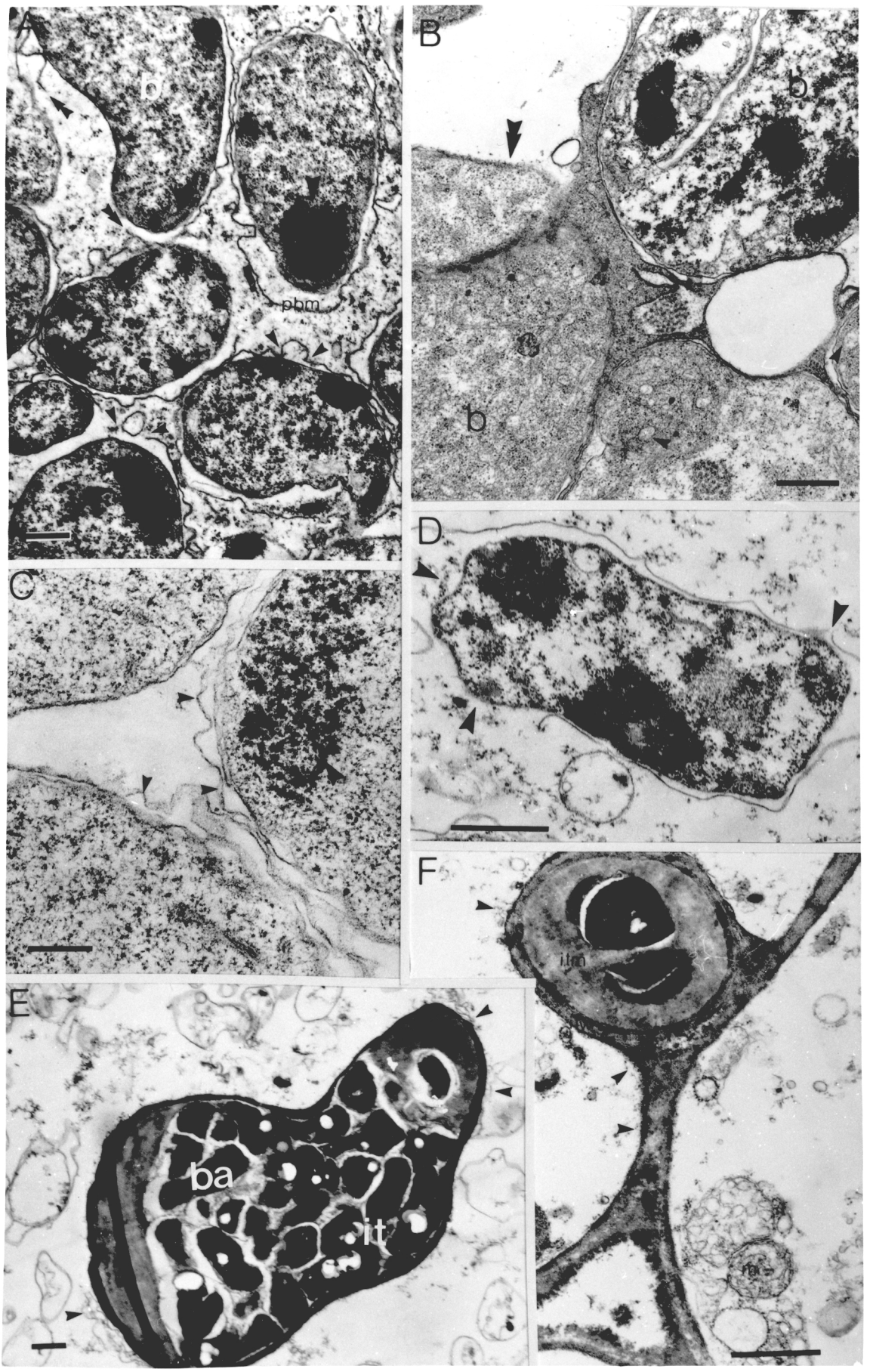

A
b
pbm
B
b
b
D
C
E
ba
it
F
itm
ba

Plate 12. Fusilade treated nodules.

A. The infection thread (it) wall becomes fibrous and disperses (arrows).

B. Vegetative bacteria (ba) are released from the degenerate infection thread (it) (arrows).

C. Vegetative bacteria (ba) proliferate in the dispersed host cell cytoplasm. cw=cell wall.

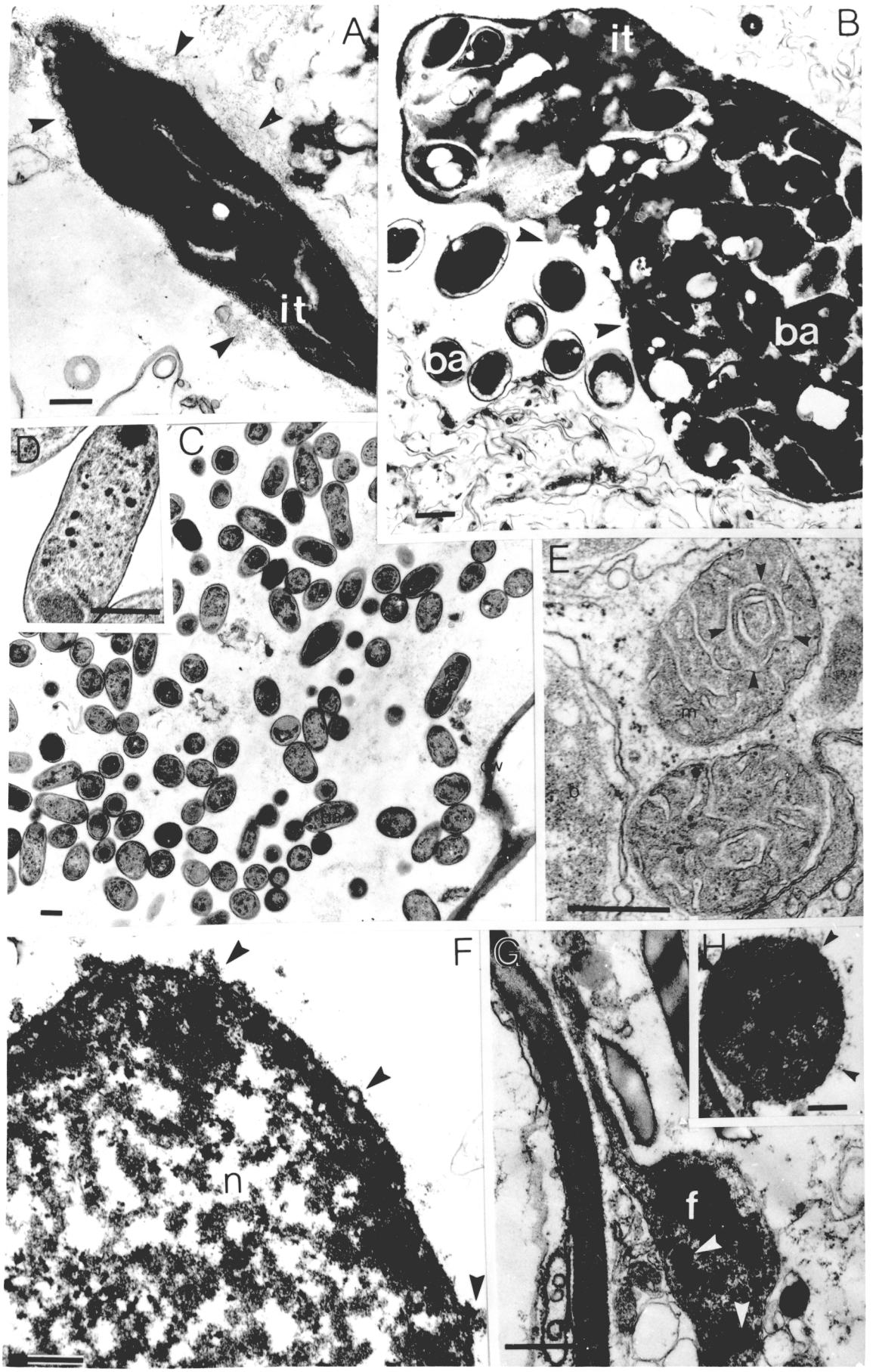
D. The bacteria within the nodule have identical structure to vegetative bacteria.

E. Mitochondria (m) exhibit extremely contorted cristae (arrows) which appear to form circles.

F. The host cell nucleus remains intact until late in the sequence of deterioration, whereupon the nuclear membrane ruptures (arrows) and releases nuclear material.

G. Plastids are often intact in degenerate cells. These contain ferritin (f) and large osmiophilic globules (arrows).

H. Plastids eventually swell, having lost membraneous integrity, becoming spherical and rupture (arrows).


A
it
B
it
ba
ba
C
D
cv
E
m
b
F
n
G
f
H

15.4.2. Mature symbiotic tissue.

Tissue approaching maturity shows signs of degeneration (Plate 11.A). The host cytoplasm becomes light and dispersed, containing fewer ribosomes than normal and little rough endoplasmic reticulum. Golgi bodies are present and are budding vesicles. Plastids and mitochondria remain along the periphery of the host cell. Plastids embody some osmiophilic plastoglobuli and small amounts of starch. Bacteriods contain many electron dense granular regions situated around the periphery of the bacteriod cytoplasm (Plate 11.A.) and peribacteriod membranes are ruptured. Many small vesicles are associated with these ruptures and appear to fuse with the peribacteriod membrane (Plate 11.A. - small arrows). Bacteriod outer membranes are indistinct in places, particularly near discontinuities of the peribacteriod membrane (Plate 11.A. - double arrows), and may be damaged allowing cytoplasmic material to escape from the bacteriod. Other areas at this stage show further deterioration, with empty host cell cytoplasm and ruptured peribacteriod membranes.

Mature symbiotic tissue is also affected by the presence of fusilade. Some bacteriods are normal in appearance, while others in the same cell are deteriorating (Plate 11.B). Intracytoplasmic vesicles appear enlarged in some bacteriods (Plate 11.B. - arrows). Deteriorating bacteriods exhibit the electron dense clumping and otherwise light and dispersed cytoplasm that is present in damaged early symbiotic bacteriods, others have shrunken cytoplasm (Plate 11.B. - large double arrow). Deteriorating bacteriods are often contorted and have discontinuous outer membranes.

Peribacteriod membranes disperse (Plate 11.C. - small arrow) and bacteriod membranes become diffuse and uneven. Electron dense clumps are still obvious in the bacteriods (Plate 11.C. - large arrow). Granular bodies are no longer present, and only occasional intracytoplasmic vesicles are visible. The bacteriod cytoplasm is light and fibrous in appearance. The host cell cytoplasmic matrix is absent. Some host cell organelles such as mitochondria persist.

Bacteriod membrane breakdown continues (Plate 11.D.) with bacteriod cytoplasmic material being released into the empty host cell cytoplasm (Plate 11.D. - arrows). Membrane fragments tend to show a circular profile.

15.4.3. Infection threads.

Infection threads may be found at late stages in deterioration (Plate 11.E). These lose their surrounding membrane, fragments of which can be seen adhering to the infection thread wall (Plate 11.E. - arrows). The infection thread wall deteriorates at the same time as the host cell wall (Plate 11.F - arrows). Wall material becomes fibrous and may be released into the empty host cell cytoplasm (Plate 12.A. - arrows) until no wall remains surrounding the infection thread (Plate 12.B). The normal vegetative bacteria enclosed in the infection thread escape and may form the basis of a population of bacteria that develops within the host cell (Plate 12.C). These bacteria are identical in morphology to free living rhizobia (Plate 12.D) but do not contain large PHB inclusions.

15.4.4. Host cell organelles.

Mitochondria in normal nodules show highly convoluted cristae. However mitochondria in nodules of plants grown in the presence of fusilade show an even greater degree of convolution, in some cases appearing almost circular (Plate 12.E. - arrows). Nuclei remain intact until host cells are empty and only then show signs of deterioration (Plate 12.F). The nuclear material becomes clumped and unevenly scattered throughout the nucleus. The nuclear envelope eventually ruptures, releasing nuclear material (Plate 12.F. - arrows).

Plastids are often found intact in the deteriorated cell (Plate 12.G). These contain large aggregates of ferritin and many osmiophilic globules (Plate 12.G. - arrows). Starch grains released from deteriorated amyloplasts are free in the host cell and are deteriorating . Plastids appear to swell (Plate 12.H) and lyse at a late stage in the deterioration (Plate 12.H. - arrows).

15.5. Effects of the herbicide Kerb on the ultrastructure of white clover nodules.

15.5.1. Early symbiotic tissue.

Early symbiotic tissue exhibited over expansion of the peribacteriod membranes in response to treatment by the herbicide kerb (Plate 13.A). Bacteriod outer membranes remain attached to the peribacteriod membrane (Plate 13.A. - arrows). However the bacteriods do not appear to be expanding at the same rate as the peribacteriod membrane, hence bacteriod outer membranes become extended and detached from the bacteriod cytoplasm. Electron transparent regions are apparent in the bacteriod cytoplasm (Plate 13.A. - double arrows).

Some bacteriods have deteriorated completely within their peribacteriod membranes. In other regions of the early symbiotic tissue the bacteriods are intact but show signs of collapsing (Plate 13.B). Bacteriod outer membranes are very uneven, causing bacteriods to be oddly shaped. Bacteriods in nodules of kerb treated plants range in size more than is normal. The bacteriod cytoplasms vary in density from very light to dense. Generally the smaller the bacteriod, the denser its cytoplasm. Bacteriod cell walls are diffuse in places. Some bacteriods appear collapsed.

Enlarged peribacteriod membranes are common in tissue approaching maturity (Plate 13.B. - arrows). Bacteriods appear to lose contact with the peribacteriod membrane as it enlarges. Bacteriods shrink, their cytoplasm becomes dense and their outer membranes loosen. Some fibrous material is detaching from the bacteriod surface into enlarged peribacteriod spaces. This may be bacteriod cytoplasmic material lost through ruptures in the bacteriod outer membranes.

15.5.2. Mature symbiotic tissue.

In some areas of mature symbiotic tissue enlarged peribacteriod membranes are a frequent symptom of kerb treatment (Plate 13.C. - arrows). Enlargement of the peribacteriod membrane is associated with loss of contact between the peribacteriod membrane and the bacteriod outer wall followed by bacteriod cell wall rupture (Plate 13.D. - arrows) and deterioration (Plate 13.E.). Bacteriod cytoplasmic material is released into the peribacteriod space through ruptured bacteriod outer membranes (Plate 13.D. - double arrows). Affected bacteriods often contain groups of electron dense inclusions in their cytoplasm (Plate 13.D). Many bacteriods are much smaller than the surrounding intact bacteriods at this stage. This appears to be a result of a lack of development of these bacteriods.

Occasionally multiple bacteriods occupy the same peribacteriod space (Plate 13.F). In these cases bacteriod cytoplasms are condensed and bacteriod outer membranes become loose and detached from the bacteriod cytoplasm (Plate 13.F. - arrows). Peribacteriod membranes are uneven and discontinuous (Plate 134.F. - double arrow). Fibrous material is present in the peribacteriod space. The host cell cytoplasm also exhibits deterioration, becoming light and fibrous, with few ribosomes, and little endoplasmic reticulum.

Plate 13. Kerb treated nodules.

A. Peribacteriod membranes expand and bacteriod outer membranes remain attached to peribacteriod membranes (arrows). Electron transparent regions are apparent in the bacteriod cytoplasm (double arrows).

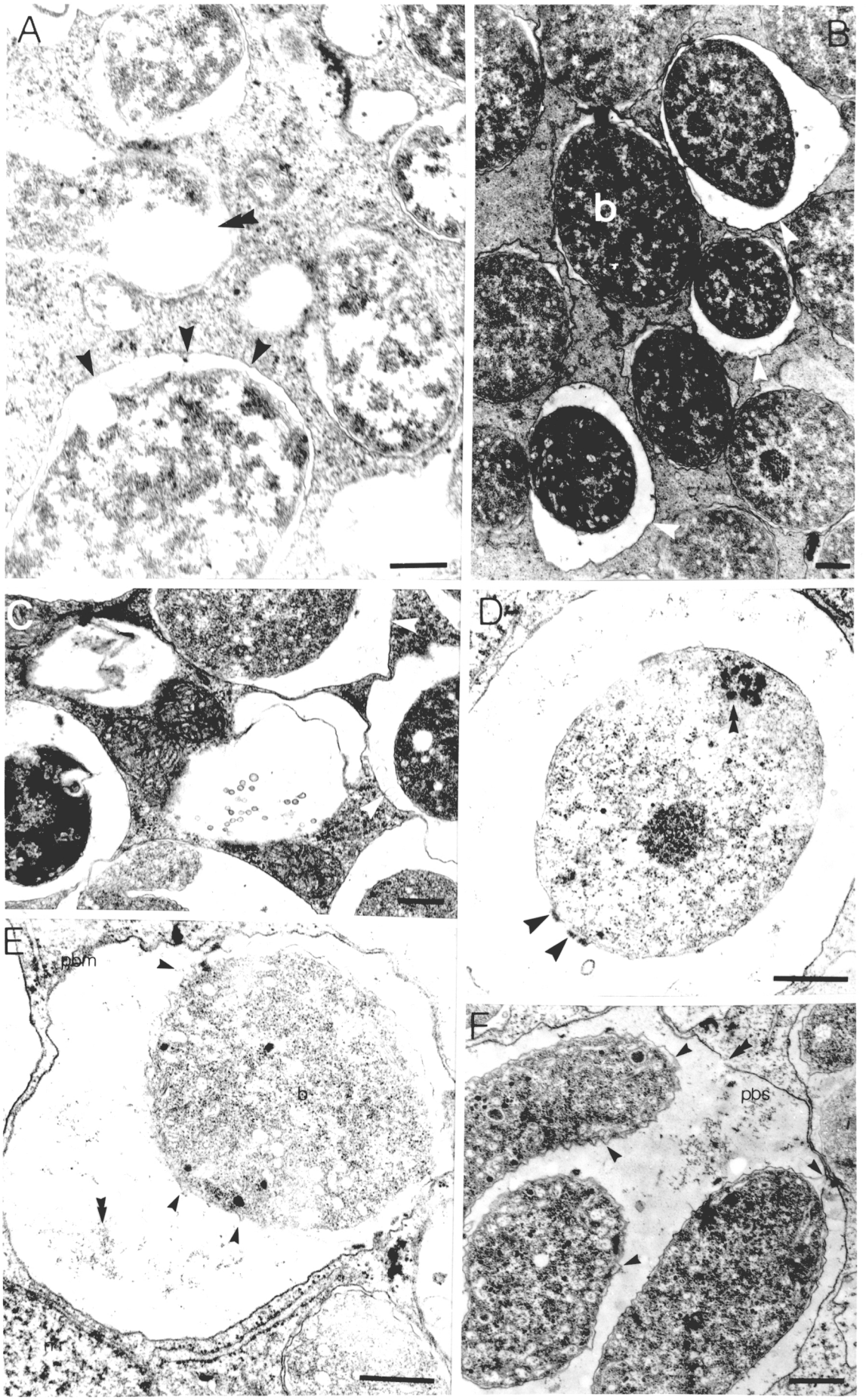
B. Bacteriods (b) in early symbiotic tissue often appear intact but show signs of collapsing, and are surrounded by expanded peribacteriod membranes (arrows).

C. Mature symbiotic bacteria have many enlarged peribacteriod membranes (arrows).

D. Bacteriod cell walls rupture (arrows). Bacteriods often contain an electron dense region (double arrow).

E. Bacteriod cell walls rupture (arrows) and fibrous cytoplasmic material is released into the peribacteriod space (double arrow).

F. Multiple bacteriods occupy the same peribacteriod space (pbs) . Bacteriod outer membranes are uneven in outline (arrows) and the peribacteriod membrane is ruptured in places (double arrow). Fibrous material is present in the peribacteriod space.


A
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pbm
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F
pbs

Plate 14. Kerb treated nodules.

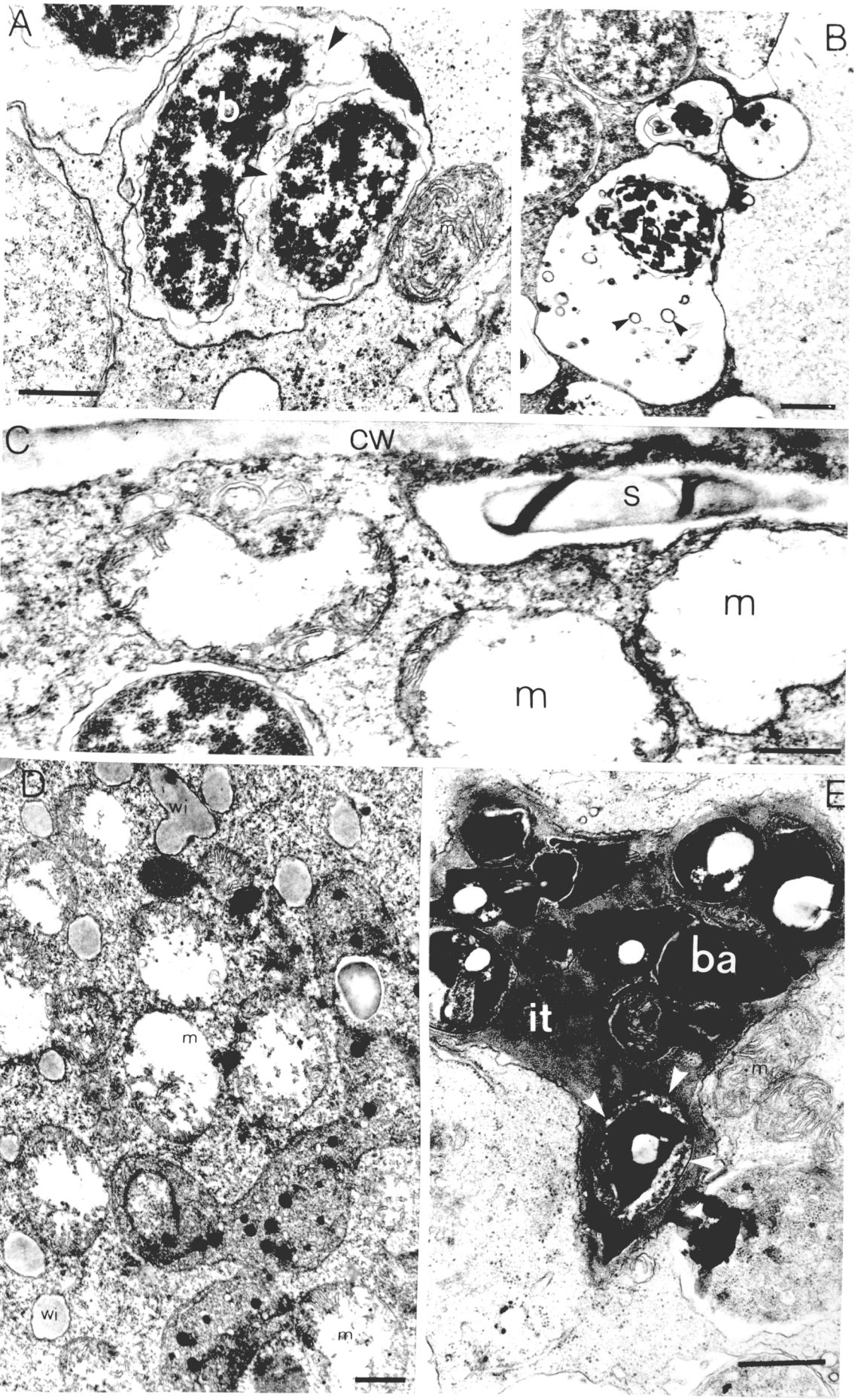
A. Bacteriod outer membranes rupture and release cytoplasmic material (arrows) into the peribacteriod space. Endoplasmic reticulum (double arrows) is enlarged with few ribosomes attached. m=mitochondria.

B. Vesicles are often present in the peribacteriod space (arrows) of deteriorating bacteriods (b).

C. Mitochondria (m) in interstitial cells are severely damaged. s=starch, cw=cell wall.

D. The matrix of mitochondria (m) in transfer cells of the pericycle is dispersed. p=plastid, wi=wall ingrowth.

E. Infection threads (it) containing bacteria (ba) are present in degenerate cells. Bacteria being released appear damaged (arrows). m=mitochondria.


A
b
m
B
C
cw
s
m
m
D
wi
m
wi
m
E
ba
it
m

Bacteriods collapse, either within their peribacteriod membranes, or following a rupture in the peribacteriod membrane (Plate 14.A). Bacteriod cytoplasm becomes highly electron dense. Bacteriod outer membranes rupture (Plate 14.A. - arrows) and cytoplasmic material is released.

Vesicles are sometimes found in the peribacteriod space associated with a deteriorating bacteriod (Plate 14.B. - arrows). As the peribacteriod space is free of fibrous material when vesicles are present, it seems likely that these vesicles are associated with breakdown of released material.

15.5.3. Host cell organelles.

Endoplasmic reticulum is present in the dispersed host cell cytoplasm and appears enlarged with few ribosomes attached (Plate 14.A. - double arrows). Mitochondria in early symbiotic cells are distributed throughout the cells, often between bacteriods. Mitochondrial matrix is very light and dispersed. Cristae are numerous and enlarged. Mitochondria in interstitial and transfer cells are particularly severely damaged, often with large regions of the mitochondrial matrix dispersed (Plate 14.C and 14.D). Dictyosomes are present in cells containing degenerate bacteriods, but these often appear not to be budding vesicles.

15.5.4. Infection threads.

Infection threads are present in damaged bacteriod filled cells of nodules of kerb treated plants (Plate 14.E). Infection threads continue to release bacteria. The bacteria in the infection thread are damaged by this herbicide treatment. The cytoplasm of emerging bacteria in the infection thread are more condensed than normal and have pulled away from the bacterial cell wall leaving empty or fibrous regions in the bacterial cytoplasm (Plate 14.E. - arrows).

Chapter 16.0. Discussion of Herbicide Effects on Nodule Ultrastructure.

16.1. Preamble.

This discussion concentrates on the interpretation of herbicide effects on nodule ultrastructure, in respect to the information that this provides on the function of cellular components in symbiotic nodule cells.

16.2.Ineffectiveness.

Ineffectiveness refers to that association between root nodule bacteria (genus *Rhizobium*) and host leguminous plants, whereby root nodules are produced but little or no atmospheric nitrogen is fixed. This can be due to genetic causes on the part of the plant or the rhizobium. Ineffectiveness can also be caused by detrimental environmental conditions on an otherwise effective association, this effect has been defined as transient ineffectiveness by Jordan (1974). Ineffectiveness induced by herbicide treatment of nodulated plants is therefore encompassed by the definition of transient ineffectiveness. The structure of selected ineffective nodules has been used to provide information on the requirements and function of nodule components.

The aim of this part of this study is to demonstrate that herbicide damage to nodules is frequently at the ultrastructural level, and is therefore often not detected at the visual or optical level.

16.2.1. Membranes.

The primary symptom of herbicide activity appears to be a general damage to membranes of both the plant and the bacteriod. Pesticides may be expected to exhibit a deleterious effect on cell membranes as these are the site of many enzyme reactions within the cell.

The quaternary ammonium salts, paraquat and diquat cause considerable damage to certain membrane systems shortly after treatment (Plate 4.A). In flax cotyledon leaves floated on paraquat ($1X10^{-4}M$) tonoplast breakdown was first observed in spongy mesophyll cells 6 hours after treatment (Harris and Dodge 1972a). These authors felt that changes following tonoplast rupture appeared to be as much due to the release of vacuolar contents, liberating hydrolytic enzymes and causing sudden osmotic change, as to the direct activity of the herbicide on cellular metabolism itself.

Tonoplast disruption due to paraquat treatment occurred prior to the final disruption of host cell organelles or bacteriods, whereas other herbicides induced degeneration of the cytoplasmic contents prior to the breakdown of the tonoplast. However early signs of disruption of bacteriods and host cell organelles was usually observed prior to tonoplast disruption (Plate 4.A) and was therefore a direct result of the herbicides on the cytoplasmic contents rather than the result of hydrolytic enzymes released from the tonoplast. Tonoplast disruption probably does play a role in the later stages of bacteriod degeneration as evidenced by the swelling and rupture of bacteriods and organelles after the degeneration of host cell groundplasm (Plate 5.B).

16.2.2. Bacteriods.

Peribacteriod membrane damage has frequently been recorded as a common feature in the ultrastructure of symbiotic cells of ineffective nodules. Peribacteriod membranes showed frequent ruptures and eventual complete breakdown following herbicide treatment (Plate 7.F,Plate 4.F, Plate 8.C, Plate 13.F).

Werner *et al.*(1980) infected *Glycine max* (soybean) with an ineffective strain of *Rhizobium japonicum*. By day 15 the peribacteriod membranes of ineffective nodules were highly irregular. Dart and Mercer (1965) found bacteria released from infection threads in transiently ineffective (NH_4NO_3 treated) nodules did not become enclosed by a peribacteriod membrane. Following defoliation (Vance *et al.*1980) or application of a surfactant fungicide (Fisher *et al.*1978) bacteriods quickly lost their peribacteriod membranes leaving bacteriods free in the host cell cytoplasm. Complete deterioration of the bacteriods followed collapse of the peribacteriod membranes in all cases. This sensitivity of the bacteriods following loss of enclosure by the peribacteriod membrane is indicative of the change in the wall of the bacteriod from its vegetative state, and the bacteriods reliance on the peribacteriod membrane for osmotic regulation and protection from host cell hydrolytic enzymes.

Peribacteriod membranes have been shown to be essential to nitrogen fixation by bacteriods (Laane *et al.*1978; Malik-Sarkisyan *et al.*1982). It is not suprising therefore, that herbicides affecting nitrogen fixation of nodules often damage the peribacteriod membrane. Alternatively damage to the peribacteriod membrane would indicate a lowering of efficiency and rate of nitrogen fixation by the nodule. Bacteriods do not lyse however, when ruptures in the peribacteriod membrane are repaired. In nodules exposed to the herbicide fusilade, mature tissue develops small discontinuities in the peribacteriod membranes which appear to be repaired by host cell vesicles (Plate 11.A) until the ruptures become extensive and repair is no longer possible.

Laane *et al.*(1978) found nitrogen fixation to be controlled by the ATP/ADP ratio within cells. The generation of reducing equivalents for nitrogenase is regulated by the energized state and the integrity of the bacteriod cell membrane. Fusilade is believed to interfere with ATP synthesis (Plowman *et al.* 1980). Damage to the peribacteriod membrane probably reflects fusilade disruption of the ATP/ADP ratio.

16.2.3.Bacteriod deterioration within peribacteriod membranes.

Peribacteriod membrane rupture is not a requirement to trigger bacteriod deterioration. Particularly within early symbiotic tissue of MCPB and fusilade treated nodules, bacteriods degenerated within their peribacteriod membranes (Plate 6.B, Plate 10.B). This deterioration appears to be often associated with lack of enlargement of the bacteriods following emergence. Unexpanded bacteriods rarely had physical contact with the surrounding peribacteriod membrane. These bacteriods in some cases exhibit normal vegetative bacterial cell wall structure, appear to divide and rapidly degenerate

(Plate 10.B). The failure of bacteriods to enlarge has frequently been observed in ineffective nodules. Bergersen (1958) described this phenomena associated with the production of a water soluble polysaccharide which accumulated between the protoplast of the infected host cell and the cell wall.

The host cell is thought to have control over bacteriod morphology as bacteriods of different *Rhizobium* strains in effective nodules of a given host nearly always take on the same morphology (Price *et al.*1984). A single strain of *Rhizobium* may also produce different bacteriod types in different hosts (Kidby and Goodchild 1966;Dart 1977). Van Brussel *et al.*(1978) postulated that alterations in bacteriod cell wall structure following release from the infection thread is involved in facilitating communication and transport of metabolites between the bacteriod and the host cell cytoplasm. It is therefore probable that non-expansion of bacteriods in herbicide treated nodule cells occurs due to breakdown in communication from the host to the bacteriod, and results in eventual starvation of the bacteriod. Tubules have been observed in the peribacteriod space of *G.max*, *P.vulgaris* and *V.unguiculata* which appear to connect the bacteriod with the peribacteriod membrane (Dart 1977). It is possible that the contact points normally in existence between the peribacteriod membrane and the bacteriod outer membrane in this symbiosis may be involved in bacteriod-host cell communication.

In red clover (*Trifolium pratense*) an ineffective symbiosis was described by Chandler *et al.*(1973) in which the bacteria are released and develop into imperfect bacteriods that remain enclosed by peribacteriod membranes. These bacteriods contained many small vesicles, were sparsely distributed and lay among untransformed bacteria. After 7 days bacteriods and host cytoplasm degenerated rapidly. Pankhurst *et al.*(1972) described a symbiosis formed by a riboflavin requiring mutant of *Rhizobium trifolii* in which the bacteria were released from infection threads but remained rod shaped in young nodules. In older cells there was an increase in infected enlarged cells in the basal region. Bacteriods were less swollen and more irregular than normal.

16.2.4. Lack of bacteriod separation.

Lack of separation of dividing bacteriods in the host cell results in multiple bacteriods enclosed by one peribacteriod membrane, this is a normal feature in some symbiotic nodule associations. However other nodules (including those of the *Trifolium repens/Rhizobium trifolii* type) characteristically contain only one bacteriod per peribacteriod membrane (Plates 1 to 3). Ineffective nodules may exhibit multiple bacteriods per peribacteriod membrane as a symptom of their ineffectiveness.

Pankhurst and Gibson (1973) described a temperature sensitive *Rhizobium trifolii* strain which formed ineffective nodules at 30^{o}C. These bacteria failed to enlarge upon release from the infection thread and remained rod shaped. The bacteria divided until there were 6 bacteriods per peribacteriod membrane, at which stage the peribacteriod membrane deteriorated releasing the bacteria into the host cytoplasm.

Ronson (1981) isolated a C_4-dicarboxylate mutant of *Rhizobium trifolii* which formed an ineffective association with *Trifolium repens*. The bacteriods were smaller than normal and also divided without separating into individual peribacteriod membranes. By 19 days after inoculation up to 7 bacteriods remained in each peribacteriod membrane in an ineffective symbiosis of *Rhizobium japonicum* with *Glycine max* (soybean). These peribacteriod membranes later disintegrated followed by the deterioration of the bacteriods (Werner *et al.*1980). Jordan (1974) described an ineffective symbiosis in which up to 6 degenerating bacteriods remained within one peribacteriod membrane. Kerb, bentazone and fusilade all induced this symptom in early symbiotic tissue to some extent (Plates 13.F, 8.D and 10.D).

Distinct contact points between the peribacteriod membrane and the bacteriod were frequently observed in normal nodule cells of *T.repens/R.trifolii* examined during this study (Plate 2.A). Robertson (1983) has suggested that contact points between the peribacteriod membrane and the bacteriod outer membrane are responsible for regulating peribacteriod membrane division and so assuring the correct ratio is maintained. However in many of the multiple occupancy cases observed in herbicide treated nodules, these contact points remained intact (for example Plate 8.D). Either these contact points are not the mechanism for maintaining the bacteriod-peribacteriod membrane ratio, or the herbicide instead interferes with their functioning, perhaps through damaging host control. Kidby and Goodchild (1966) postulated that the peribacteriod membrane development is under host control. This hypothesis was later supported by evidence of Pankhurst and Gibson (1973).

Growth of nodulated plants in the presence of the herbicides MCPB, fusilade and kerb appeared to affect bacteriod development without damaging peribacteriod membrane growth during the early stages of degeneration (Plates 6.B, 10.E and 14.B). Kerb treatment caused a particularly dramatic demonstration of this effect with the bacteriod outer membrane remaining attached to the enlarging peribacteriod membrane (Plate 14.B). As the bacteriod itself was not enlarging, the bacteriod membrane becomes detached from the bacteriod cytoplasm. This tends to support the hypothesis that the peribacteriod membrane is not controlled by the bacteriod and is, therefore, under host cell control.

16.2.5. Lack of bacterial release.

Bergersen (1957) described an ineffective symbiosis in *Trifolium subterraneum* in which the bacteria are not released from infection threads. Truchet *et al.* (1980) found inoculation of lucerne seedlings with a leucine requiring mutant of *Rhizobium meliloti* also resulted in the formation of ineffective nodules in which the bacteria were not released from the infection thread. In none of the herbicide treatments studied was bacterial release inhibited. Kerb treatment caused some damage to bacteria about to be released, but bacteria more deeply embedded in the infection thread were unharmed. This may indicate these bacteria are already altering toward bacteriod structure prior to release.

Pankhurst (1974) hypothesized that the bacterial cell wall was the most likely site for defects leading to ineffective nodulation. However he was unable to correlate antibiotic or metabolic mutations to the type of structural defect observed.

16.2.6. Host cell structural alterations.

Paraquat and fusilade induced the formation of an extremely dense host cell cytoplasm in symbiotic nodule cells. Dilated E.R. was observed in cells exposed to MCPB, fusilade and kerb. Disrupted mitochondria were frequently found following kerb, MCPB and paraquat treatments. Mitochondria also often showed signs of deterioration after treatment of plants with fusilade and bentazone. All of these symptoms have been observed in genetically ineffective nodules. In a *Medicago sativa/Rhizobium meliloti* mutant symbiosis deterioration of bacteriods was accompanied by a build up of RER in the host cells. There was also an increase in the number of mitochondria (Jordan 1974).

Ineffective bacteriodal cells of *G.max* showed an extremely electron dense cytoplasm, dilated RER with stained contents and disrupted mitochondria (Bassett *et al.*1977b). Mature ineffective nodules of *G.max* were found to contain shortened mitochondria and less contact to amyloplasts than normal (Werner *et al.*1980). Rolfe *et al.*(1980a) demonstrated an ineffective association between *Trifolium repens* and *Rhizobium trifolii* which resulted in large vacuolate cells with no bacteriods or bacteria present although there were clusters of cells with rhizobia. Lakshmi-kumari *et al.*(1974) observed pseudonodules on roots of Medicago sativa treated with amitrole, these nodules had no rhizobia in the tissue. Low levels of bentazone present from seed germination caused a similar symptom of nodules without symbiotic tissue, although some cells did contain rhizobia.

The nucleus is thought to be resistant to herbicide treatments, not usually being affected until cells are severely degenerate and then the effect is probably one of senesence rather then a specific effect of the treatment (Anderson and Thomson 1974). Symbiotic cell nuclei exposed to paraquat did show early signs of damage (Plate 4.C), becoming extremely electron dense and therefore indicating a direct effect on the nodule by this herbicide.

Dodge (1971) observed swelling and disruption of mitochondria in paraquat treated flax leaves. The rupturing followed breakdown of the tonoplast and was attributed to sudden changes in osmotic conditions rather than a direct effect of the herbicide on mitochondrial membranes. Mitochondria in nodules exposed to paraquat showed considerable damage in cells containing bacteriods and an intact tonoplast. Hence the effect of paraquat appears to act directly on the mitochondria of nodules rather than indirectly via tonoplast disruption.

16.3. Selective autolysis.

Bassett *et al.*(1977b) described an ineffective symbiosis of *G.max* in which the bacteriods degenerated within their peribacteriod membranes. By 21 days after inoculation only traces of bacteria remained and the host cell contained many empty peribacteriod membranes. This process was similar in some ways to senesence, however some host cell organelles, such as mitochondria and plastids, remained intact. This effect was labelled by the authors "selective autolysis". It was suggested that this phenomena may be a host cell response to normal infection by a pathogenic microorganism, ie the bacteria had lost the ability to inhibit host cell defenses. Bassett *et al.*(1977b) offered two possible explanations for deterioration by selective autolysis. Firstly the presence of an acute localized nitrogen deficiency due to the bacteriod being unable to fix nitrogen may cause breakdown of the symbiotic association. Alternatively the bacteriod cell wall changes at the time of release from the infection thread may release antigens. In the ineffective nodule these antigens may not be recognized by the host cell and could trigger an incompatible reaction from the host plant. The ensuing selective autolysis was suggested to be an attempt by the plant to destroy an incompatible invader, in a similar way to the hypersensitive response where an invaded plant destroys its own tissue in order to halt invasion by a pathogen. Werner *et al.*(1980) later demonstrated that lack of nitrogenase activity does not necessarily trigger dissolution of the bacteriods.

A parallel hypothesis may be drawn for the effects observed in nodule cells affected by fusilade and bentazone. In these cases host organelles were present in cells in which bacteriods had degenerated (Plate 9.D and 12.G). For example, degenerated bentazone-treated cells had no intact bacteriods although free ribosomes, budding dictyosomes, mitochondria, membraneous remains and starch released from amyloplasts were present and often aggregated around the intact host nucleus.

Deteriorating cells in nodules treated with fusilade often contained plastids, mitochondria and large starch grains free from amyloplasts at the periphery of degenerate cells. Peribacteriod membrane fragments are also frequently present in these degenerate cells- these membranes are believed to be of host cell origin (Goodchild and Bergersen 1966). Herbicide treatment may interfere with plant cell recognition of the bacteriod cell wall antigens, or the bacteriod cell wall antigens themselves, causing the plant to attempt to destroy the rhizobia as it would an invading pathogen.

16.3.1. Bacteriods.

Bacteriods in symbiotic tissue of nodules exposed to herbicides frequently showed distinctive early indications of damage. Early symbiotic tissue in nodules exposed to fusilade contained bacteriods whose cytoplasm formed dense aggregates of material at the periphery of the bacteriod cytoplasm (Plate 11.A). These regions appear to be formed by the accumulation of bacteriod cytoplasmic material following degeneration of the bacteriod cytoplasmic matrix. Other herbicides also induced this symptom although usually at later stages of degeneration. This symptom may be

comparable to that found by Dart and Mercer (1965) who noted a blotchy appearance of the bacteriod cytoplasm in nodules of plants treated with NH_4NO_3.

Bentazone treatment induced extensive intracytoplasmic vesicles in bacteriods of mature symbiotic tissue (plate 8.D). Dart and Mercer (1965) suggested that these vesicles are the site of oxidation-reduction reactions, and may be involved in supplying electrons for nitrogen fixing activity. Gourret and Fernandez-Arias (1974) postulated the same function for these vesicles after finding they were positively stained by diaminobenzidine (DAB). An increase in the number of these vesicles may indicate a blockage in activity of oxidation-reduction reactions and a concomitant attempt to compensate for the lowered efficiency. Therefore bentazone may affect bacteriod function by blocking oxidation-reduction reactions.

16.4. Premature senesence.

Electron microscope examination of pesticide treated plant tissues, in general, have revealed that the terminal changes associated with herbicide induced plant death are similar, if not identical, to ultrastructural changes associated with the final stages of senesence (Anderson and Thomson 1974). However fundamental differences exist. Not only is the time sequence considerably more rapid, but the order of events differs.

In the unique situation of a meristematic nodule, senesence normally occurs to aged cells in the basal portion of the nodule. During herbicide induced degeneration greater regions of the nodule become senescent, including tissue that has not yet become mature. this type of deterioration is termed premature senesence (Vance *et al.*1980;Hrabak *et al.*1985).

16.4.1. Normal senesence.

In the case of *Trifolium repens* nodules the host cell and the bacteriods are thought to senesce at the same rate (Gourret and Fernandez-Arias 1974). A similar situation has been reported for *G.max* (Tu 1974b). However Newcomb (1976) reported that the host cell deterioration preceded that of the microsymbiont in nodules of *Pisum sativum* (pea). He recorded that the earliest stage of senesence was characterized by an increasing electron density of bacteriod containing cells. At a later stage the plant cytoplasm became empty and featureless, while the peribacteriod membranes and bacteriods remained unaffected until the final stages.

Tu (1974) reported that the first sign of senesence was membrane damage, with the membranes of the host cell and bacteriod (the peribacteriod membrane) breaking down first followed by degeneration of organelle membranes. Following membrane degeneration host cell cytoplasms deteriorate, endoplasmic reticulum fragments and vesiculates, ribosomes reduce in number and eventually disappear. Also starch disappears and mitochondria swell (Sutton 1983).

16.4.2. Ultrastructure of premature senesence.

During herbicide induced premature senesence ultrastructural changes similar to those in normal senesence are found. The host cell cytoplasm of early symbiotic cells becomes electron dense when nodules were exposed to paraquat and fusilade (Plate 4.A and 10.D). However this symptom was not found when tissue was grown in the presence of kerb or bentazone, and MCPB treated tissue became light and dispersed (plate 6.E), the groundplasm appearing to have degenerated which may be equivalent to the later stages of the degeneration described by Newcomb (1976).

Plastids in early symbiotic tissue exposed to MCPB and fusilade accumulated osmiophilic globules and phytoferritin and inner membranes become invaginated, in a similar way to senescent organelles (Plates 7.E and 11.F). Swelling of mitochondrial cristae occurs, and some damaged mitochondria are found in MCPB treated tissue (Plate 6.A). Mitochondria become dense in early symbiotic cells following fusilade treatment, and finally swell at late stages of deterioration (Plate 10.D and 7.E).

Mitochondria are severely damaged by paraquat (Plate 4.B), but remain undamaged until the final stages of breakdown in tissue exposed to bentazone, being present while saprophytic rhizobia invade the empty host cells (Plate 9.B).

Mitochondria in transfer cells in the pericycle of nodules grown in the presence of kerb show severe damage (Plate 14.D). Kerb is absorbed by plant roots and is translocated in the apoplast, which may explain why cells surrounding the vascular bundle are particularly damaged. Mitochondria in the symbiotic tissue of the same nodules lose their usual position at the periphery of the host cell and are found distributed among the bacteriods (Plate 13.C). The peripheral location of mitochondria is thought to be due to the higher oxygen requirement of host cell organelles compared to bacteriods (Robertson *et al.*1983). Therefore loss of the peripheral location indicates a breakdown in control of oxygen tension in the symbiotic cell.

Hrabak *et al.*(1985) postulated that an ineffective nodule could be formed by insufficient legheamoglobin production causing decreased oxygen flux into nodules and resulting in oxygen limitation of bacteriod tissue and accelerated senesence. Nitrogenase proteins are irreversibly inactivated in the presence of oxygen and it seems probable that any variation in oxygen concentration within the symbiotic cells would result in decreased efficiency of nitrogen fixation.

Fragmentation and vesiculation of ER occurred in degenerating symbiotic cells of MCPB treated nodules (13764B), with many vesicles free in the host cytoplasm and associated with degenerating bacteriods (Plate 7.C). Truchet and Coulomb (1974) noted the presence of many vesicles in symbiotic nodule cells of *Pisum sativum* during senesence, which they labelled phytolysosomes. They believed these vesicles to be involved in the death of the bacteria and the host cell through the release of hydrolytic enzymes. Dart and Mercer (1965) found when *Trifolium subterraneum* and *Medicago tribuloides* were treated with 11mM NH_4NO_3 mature infected nodule cells showed a marked increase in the frequency of small membrane bound vesicles in the host cell cytoplasm. MCPB therefore appears to induce senesence in nodule cells.

Gourret and Fernandez Arias (1974) found in late symbiotic/early senescent nodules of *Rhizobium trifolii/trifolium repens*, bacteriods contained large PHB globules which were released during senesence. Accumulation of PHB was observed in this study during late symbiosis, however it was not observed during senesence of normal tissue, and only very rarely in herbicide treated nodules. The lack of accumulation of PHB during senesence of herbicide treated tissue is due to the premature nature of the deterioration, prior to the stage where this accumulation normally occurs It has been suggested that PHB functions as a bacteriod reserve material during photosynthate deprivation, however during seasonal or nitrate induced nodule senesence PHB levels stay constant or increase (Sutton 1983). I believe PHB accumulation may be a symptom of waste accumulation due to cessation of transportation of metabolites into the host cell. Infection threads are present in

senescent cells of normal tissue and these also were present in herbicide induced senescent cells (Plates 11.E, 11.F, 5.B and 8.E).

Dodge (1971) concluded that in whatever way the process is initiated, plant cell death is certain following tonoplast disruption. This is also the case in symbiotic cells of herbicide treated plants. However the irreversible point in deterioration probably occurs prior to tonoplast disruption in nodule cells, as bacteriods and host cell cytoplasm in some cases show considerable disruption prior to tonoplast disruption.

16.5. Defoliation vs. herbicide effect.

Paraquat is known to halt photosynthesis very rapidly by competing for electron flow from the primary electron acceptor of photosystem I (Dodge 1971). It could therefore be expected that paraquat damage of nodule structure would be an indirect one through halting the flow of photosynthetic products to the nodule. Comparisons to defoliation experiments are therefore valid.

Vance *et al.*(1980) described the effects of 70-80% defoliation on the ultrastructure of nodules of *Medicago sativa*. The degeneration of nodule tissue due to defoliation was found to be similar to a process of premature senesence. Membranes of the plant and bacteriod deteriorated and high levels of vesiculation were found in the host cytoplasm, which became light and dispersed.

Deterioration of early symbiotic tissue following paraquat treatment is very different from normal senesence (Plate 4). The host cell cytoplasm became dense, vesiculation was not observed and host cell organelles and cytoplasm deteriorated prior to the bacteriods. Membranes of both the bacteriod and the plant cell appeared to be the primary target of the damage although bacteriods were degenerate prior to peribacteriod membrane breakdown. In mature symbiotic cells deterioration of the host cell cytoplasm preceded that of the bacteriods. In late stages of degeneration infection threads are present and surrounded by a membrane continuous with the plasmalemma of the host cell.

The plasmalemma has been recorded as being the last cell structure to deteriorate in normal senesence of plant cells (Anderson and Thomson 1974). The peribacteriod membrane enclosing the bacteriods arises by an endocytotic process as the bacteria pass out of the infection thread (Dixon 1967; Bassett 1977). Further growth of this membrane is achieved by fusion of vesicles of host cell origin (Robertson and Lyttelton 1982). It is therefore suprising that the peribacteriod membrane is completely degenerated in paraquat treated nodules while the infection thread membrane and plasmalemma remain intact (Plate 5.B). The implication of this result is that peribacteriod membranes alter considerably following detachment from the infection thread membrane. This evidence also supports the belief that infection threads and their membranes are extensions of the plant cell wall and plasmalemma (Callaham and Torrey 1981).

Chapter 17. Conclusions.

17.1. Testing on *Rhizobium trifolii.*

In vitro testing of herbicides commonly used in agriculture against weeds of white clover crops indicated that paraquat and MCPB have the potential to affect growth of *Rhizobium trifolii* when applied at concentrations higher than those recommended for application in the field. However alternative testing methods in broth culture showed no effect of any of the five herbicides under test on growth of *R.trifolii.* It is probable that this indicates the growth of *R.trifolii* in the field would not be affected by levels of these herbicides that normally contact this bacterium. However sub-bacteriostatic doses of phenoxy-herbicides have been found to reduce the ability of *R.trifolii* to form a symbiosis with clover (Fletcher and Raymond 1956). Therefore the possibility that these herbicides may also act in this way cannot be completely eliminated.

17.2. Testing on *Trifolium repens.*

White clover (*Trifolium repens*) was extremely sensitive to herbicide application at early stages of growth under *in vitro* conditions. This result indicates there is danger in planting clover soon after a previous crop has been chemically cleared.

17.2.1. Paraquat.

Of the 5 herbicides tested, paraquat exhibited the most toxic effects both *in vitro* and *in vivo*, causing severe dessication and decreasing nodulation. This herbicide acted more severely against nodulation than against plant weight in both *in vitro* and *in vivo* experiments, indicating that nodules are particularly sensitive to paraquat treatment. Ultrastructure of paraquat treated nodules differed from that of nodules of defoliated plants as described by Vance *et al.*(1980). Thus paraquat does not affect nodules solely by loss of photosynthates following plant defoliation and is probably exerting a direct effect on nodules. The nodules used for ultrastructural examination were excised from plants treated with the herbicide *in vitro* and were therefore directly exposed to paraquat. In the field paraquat is applied foliarly and is known to bind tightly to soil colloids, the actual levels of paraquat contacting nodules *in vivo* are therefore much lower than that actually applied. This effect was apparent from a comparison of *in vitro* and pot experiments, where particularly root tissue, but also weight of plants treated in pots was not as severely affected by paraquat as occurred *in vitro.* Also plants grown in soil maintained at higher water levels were more damaged by paraquat than plants grown in drier soil, presumably as paraquat was more available to plant roots when the soil was wetter.

Paraquat is known to damage chloroplasts severely by competing for electron flow from the primary electron acceptor of photosystem I (Harris & Dodge 1972a). It is probable that damage to nodules would result to some degree from loss of photosynthates necessary to nodule functioning. However ultrastructure of nodules

showed direct activity of paraquat particularly on membranes within symbiotic host cells and bacteroids, indicating paraquat also blocks electron flow in these cells.

17.2.2. MCPB.

MCPB inhibited growth of *R.trifolii* on solid medium but did not affect growth of this bacterium in broth culture. It is therefore unlikely (but not impossible) that this herbicide would affect growth of *R.trifolii* in the field, as herbicide levels contacting bacteria in soil would be lower then those *in vitro*.

Both *in vitro* and pot experiments on plants indicated that the MCPB tested was probably not pure. It may have contained either a small amount of the active form of MCPB (MCPA), or MCPB was being broken down to MCPA by the rhizobia, since white clover has been shown to be unaffected by pure MCPB (Fletcher *et al.*1956). Root growth of white clover was extremely stunted following treatment with MCPB and resulted in an inhibition of nutrient and water uptake by roots. This herbicide was also more active against vigorously growing plants, indicating a meristematic site of activity. Sections of stunted lateral root apices show several meristems but little cell elongation. The lack of stimulation of growth following rhizobial inoculation of MCPB treated plants, and the lack of a significant effect of MCPB on nodule numbers *in vitro*, indicated that MCPB interfered with nodule activity. Ultrastructural observations of MCPB treated nodules showed that MCPB had a severe effect on bacteriods and host cell mitochondria. Nodules also had high levels of vesiculation and dilated endoplasmic reticulum. Audus (1964) reports that MCPA prevents immature cytoplasm from changing into mature cytoplasm and mature cytoplasm seems to revert to the immature state. Immature or active tissue normally has higher levels of vesiculation than mature tissue (Robards 1974). Hence high levels of vesiculation in MCPB treated tissues indicate a high level of activity similar to that of immature tissues. Vesicles in nodules of MCPB treated plants are frequently associated with degeneration of the bacteriods. Perhaps this activity is not unlike that of hypersensitivity of plants infected with a pathogen, in that the host actively destroys the infected tissue in response to an external stimuli.

17.2.3. Bentazone.

Bentazone caused a slight inhibition of growth when applied at high concentrations to *R.trifolii* in wells on solid agar. No effect on growth of this bacterium was caused by bentazone application to broth culture. It is therefore improbable that bentazone would affect growth of *R.trifolii* in the field.

Bentazone did not cause growth inhibition of white clover plants and in some instances stimulated plant growth and nodulation, which is possibly due to breakdown of this herbicide releasing bound nitrogen. However in pot experiments bentazone did cause significant decreases in shoot growth and nodulation, particularly under conditions of low soil moisture. It appears that bentazone toxicity is increased by interaction with the soil environment. Alternatively bentazone activity may be lowered or breakdown increased under wet conditions. Ultrastructural observations of nodules

suggests that bentazone interfered with dispersal of bacteria through nodule tissue as bentazone treated nodules contained little symbiotic tissue. However many bentazone treated nodules were normal in this respect. Often bacteriods within these treated nodules showed an increase in intracytoplasmic vesicles. As these vesicles are thought to be the site of oxidation-reduction reactions (Dart & Mercer 1965;Gourret & fernandez-Arias 1974), this increase in vesicles may be related to the stimulation in nitrogenase activity observed *in vitro* when bentazone was applied shortly after germination. The fact that this stimulation was not apparent in plants treated 21 days after germination indicates that a breakdown of the herbicides over time is possibly required to cause this effect.

17.2.4. Fusilade.

Fusilade caused small zones of growth inhibition of *R.trifolii* when applied to agar in wells. No growth inhibition was caused by this herbicide when applied to *R.trifolii* growing in liquid culture. this variation in result is probably due to the altered growth of bacteria in the very differnet environments of solid and broth medium. It is therefore improbable that this herbicide would inhibit growth of *R.trifolii* in the soil environment.

Fusilade had varying effects on white clover growth and nodulation when tested *in vitro*, particularly inhibiting nitrogenase activity even though it stimulated nodulation and did not affect plant weight in some circumstances. Fusilade appeared to affect nodule activity directly rather than affecting nodules via damage to other plant parts. Ultrastructure also indicated a direct effect of fusilade on nodule tissue, particularly on peribacteriod membranes and the bacteriods themselves. Fusilade is believed to act on target plants by inhibition of ATP synthesis (Plowman *et al.*1980). Laane *et al.*(1978) found the generation of reducing equivalents for nitrogenase is regulated by the energized state and the integrity of the bacteriod cell membrane. Damage to the peribacteriod membrane observed after fusilade treatment, the sensitivity of nitrogenase to this herbicide and the greater activity of fusilade against plants of higher nutritional status indicates that fusilade is exerting a effect on white clover growth and nodulation by inhibition of ATP generation. Multiple occupancy of peribacteriod membranes following fusilade treatment may indicate these membranes are inhibited in their division due to lack of essential ATP.

17.2.5. Kerb

Kerb showed very low toxicity to *R.trifolii in vitro*. 10 x concentration of kerb applied to wells in the agar formed inhibition zones of less than 1mm, while all other *in vitro* applications of kerb had no effect on growth of this bacteria.

White clover *in vitro* was extremely sensitive to kerb treatment particularly when applied at the seedling stage. Plants treated at later stages of growth were not as severely affected, and in some cases high levels of kerb stimulated plant growth. Additives to the active ingredient may play a role in this activity. Kerb is an amide herbicide and it is probable that breakdown of high levels of this chemical releases

enough nitrogen to cause a growth stimulation. This hypothesis is supported by the results of pot experiments where recommended levels of the herbicide were toxic while 10 x the recommended levels were not. Nitrogen released from this herbicide could cause a growth stimulation masking toxic effects at high levels. The effect of kerb on nitrogenase activity *in vitro* mirrored the activity of this herbicide on nodules, indicating an inhibition of nodule initiation or establishment rather than of nodule activity. However the effect on nodulation did not correlate with kerb activity on plant growth *in vitro*, hence the decrease in nodulation was not due to a loss of photosynthates but was a direct effect on nodulation. Pot experiments indicated that changes in nodulation reflected changes in plant weight and was therefore due to a loss of photosynthates from the host plant. Translocation of kerb from leaves was found to be not appreciable by Carlson *et al* (1975) while it is believed to be readily absorbed by roots. Therefore differences in result between *in vitro* and pot experiments is probably due to pot applications of herbicides being foliarly applied while *in vitro* the herbicide directly contacted the whole plant.

Kerb is thought to act on target plants by inhibiting cell division and growth (Carlson *et al*.1975) Investigation of nodule ultrastructure indicated that kerb affected bacteriod development to a greater extent than development of the host cell although host cell organelles and particularly mitochondria of pericycle cells showed severe damage following kerb treatment, indicating this herbicide gains access to nodules through the apoplast, and does not only affect nodules by loss of photosynthates. Mitochondria were particularly sensitive to kerb, hence kerb may act through inhibition of mitochondrial activity in target plants.

17.3. Concluding comments.

All of the herbicides tested exhibited the potential to affect white clover growth either directly, or by inhibition of the activity of this plant's symbiosis with *Rhizobium trifolii*. Whether the activity observed here is due to the active ingredient or additives of the herbicide formulation was not tested here but may be elucidated by further research. Testing of MCPB to determine purity, and examining the possibility of breakdown of MCPB to MCPA by Rhizobium species would be of interest, as this effect would have serious effects on legume activity in the field.

Investigations into breakdown of bentazone and kerb under soil conditions particularly by soil microorganisms, would clarify whether these herbicides release nitrogen when degenerating and thereby stimulating plant growth.

Concentrations of herbicides applied here are similar to those applied in practice. However the situations under which the toxicity of these herbicides has been tested do not mirror the field environment accurately enough for these results to be extrapolated to agricultural situations directly. Often clover is treated at a later, and more resistant, stage of growth. Recovery of plants following treatment with these herbicides is another possibility that remains to be explored.

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Appendix 1. Medium.

1.Rhizobial medium.

RGM 35 minimal media per litre;

N⁻B5 stock	100ml
Iron chelate	0.5ml
Trace elements	1.0ml
Vitamins	10ml
KNO3 (10mM)	1.01g
Glucose	9g (in 100ml distilled water. Autoclave separately.)
Distilled H_2O	785.5ml
pH	6.5
Agar	12g

RGM 36 complete medium = RGM 35 + 1g/l Casamino acid + 1g/l Yeast extract.

BMM (Bergersens Medium) per litre;

$Na_2HPO_4 \cdot 12H_2O$	8ml of 45 g/l stock
$MgSO_4.7H_2O$	8ml of 10 g/l stock
$FeCl_3.6H_2O$	0.15ml of 20 g/l stock
$CaCl_2.2H_2O$	1ml of 40 g/l stock
Thiamine	1ml
Biotin	1ml
Trace elements	1ml
Distilled H_2O	980ml
Sodium Glutamate	0.5g
Mannitol	3.0g
Yeast extract	0.5g
agar	15g

2.Plant growth medium.

FMNO3 per litre;

stock solutions;

KH_2PO_4	4g/l
$Na_2HPO_4.12H_2O$	6g/l
$MgSO_4.7H_2O$	4.8g/l
$CaCl_2$	4g/l

1. 500ml dist water
2. 25ml of each stock solution
3. 5ml of iron chelate
4. 3ml of trace elements
5. 392 ml of distilled water
6. 0.5g KNO_3 (5mM KNO_3)
7. Agar 12g
8. pH 6.5

FM = FMNO3 without KNO_3.

3.Stocks.

Thiamine. 100 mg/ml of distilled water.

Biotin. 10 mg/ml of distilled water.

N⁻B5.(Nitrogen free B5 stock).

To prepare 1 litre of 10x N⁻B5 stock, dissolve the following in 800mls of distilled water;

KCL	5g
$NaH_2PO_4.2H_2O$	1.5g
Na_2SO_4	1.5g
$MgSO_4.7H_2O$	2.5g

Then dissolve 1.5g $CaCl_2.2H_2O$ in 199ml of distilled water and add. Then add 1ml of 100x stock of KI (0.75g/100ml stock of KI).

Iron Chelate. 200x stock.

$FeSO_4$	0.557g (or 0.823g of $FeSO_4.7H_2O$)
Na_2EDTA	0.745g
Distilled H_2O	100ml.

Heat to dissolve.

Trace Elements. 1000x stock.

$MnSO_4.H_2O$	100mg
H_3BO_3	30mg
$ZnSO_4.7H_2O$	30mg
$Na_2MoO_4.2H_2O$	2.5mg
$CuSO_4.5H_2O$	2.5mg
$CoCl_2.5H_2O$	2.5mg
Distilled H_2O	100ml

Vitamins. 100x stock.

Meso myo-inositol	1000mg
Thiamine HCl	100mg
Nicotinic acid	10mg
Pyridoxine HCl	10mg
Distilled H_2O	100ml.

4.Soil used in pot experiments.

Temuka Ah. Gley soil- a silty clay loam.

pH (H_2O)	5.9
Soluble salts(%)	0.34
C:N	7.9
% C	4.50
% N	0.57
Cation Exchange Capacity	
(at pH 7.0)	27.4 (m.e.%)
Calcium (m.e.%)	19.5
Magnesium "	3.8
Potassium "	3.3
Sodium "	0.1
Base saturation	97 %
Phosphorous	
Retention	24 %

Appendix 2. Growth of Rhizobia in broth culture containing herbicides.

Values represent the average of 2 replicates of each treatment.

Growth on Viable Plate Counts are X 10^5 cells/ml.

Optical Density was measured with a Bausch and Lombe Spectronic 20.

			Time (hours).				
Herbicide	Level	Count Method	0	4	24	48	72
Paraquat	10	Plate Counts	0.56	1.69	10.4	1970	12100
		Optical Density	-	-	.0105	.63	1.06
Paraquat	1	Plate Counts	0.45	1.69	8.13	1640	12600
		Optical Density	-	-	.026	.955	1.07
MCPB	10	Plate Counts	0.49	1.42	9.7	1980	9100
		Optical Density	-	-	.019	.825	.895
MCPB	1	Plate Counts	0.43	0.88	5.1	1995	8950
		Optical Density	-	-	.02	.80	.99
Bentazone	10	Plate Counts	0.475	1.37	9.25	2140	8400
		Optical Density	-	-	.014	.83	1.02
Bentazone	1	Plate Counts	0.55	1.5	8.3	-	14500
		Optical Density	-	-	.026	1.095	1.18
Fusilade	10	Plate Counts	0.48	1.64	6.39	1920	-
		Optical Density	-	-	.023	.91	1.05
Fusilade	1	Plate Counts	0.44	1.22	7.5	430	-
		Optical Density	-	-	.022	1.4	1.055
Kerb	10	Plate Counts	0.51	1.25	5	2500	10400
		Optical Density	-	-	.035	1.01	1.03
Kerb	1	Plate Counts	0.49	0.69	8.77	1980	13300
		Optical Density	-	-	.025	.78	1.18
Controls.							
Water		Plate Counts	0.48	1.335	7.89	1780	5100
		Optical Density	-	-	.016	.71	1.08
Acetone	1	Plate Counts	0.54	1.05	10.88	2140	8400
		Optical Density	-	-	.0115	.72	1.1
Acetone	10	Plate Counts	0.505	-	7.25	1700	7000
		Optical Density	-	-	.01	.64	1.17

- = growth was not recorded.

Levels.

1 = Level of herbicide application equivalent to that recommended by the manufacturers.

10 = Level of herbicide application equivalent to ten times that recommended by the manufacturers.

Appendix 3. Data from *in vitro* study of herbicide toxicity toward *Trifolium repens/Rhizobium trifolii* symbiosis.

Herbicide	Conc.	Time.	N	R	Reps	Average Shoot ht.(mm).	Average Root lgth. (mm).	Average Leaf no. per plant.	Average no.of Lateral Roots.
Paraquat	10	3d	+	+	12	3	20.25	0.33	0.00
			+	-	6	2.33	16.83	0.00	0.00
			-	+	14	2.57	21.5	0.14	0.00
			-	-	6	1.83	18.83	0.00	0.00
Paraquat	10	3wk	+	+	6	67.83	74.33	3.83	6.83
			+	-	6	62.17	62.17	3.67	7.67
			-	+	6	22.17	40.0	2.50	3.50
			-	-	6	9.0	30.17	2.00	3.67
Paraquat	1	3d	+	+	12	5.33	212.58	0.67	0.42
			+	-	6	2.5	22	0.33	0.00
			-	+	14	5.57	22	1.00	0.93
			-	-	6	2.67	22	0.33	0.00
Paraquat	1	3wk	+	+	6	68.5	72.67	4.00	7.67
			+	-	6	69.5	69	4.50	9.17
			-	+	6	25.17	54.17	2.67	3.83
			-	-	6	7.17	28.17	1.17	2.50
MCPB	10	3d	+	+	12	24.42	24.08	2.92	5.67
			+	-	6	31.83	23	3.33	6.17
			-	+	15	8.73	23.67	1.60	5.27
			-	-	4	6	22.25	1.25	5.00
MCPB	10	3wk	+	+	6	72.33	65.83	3.67	6.00
			+	-	6	72	83.83	4.00	6.50
			-	+	6	13	41.67	1.67	2.83
			-	-	6	12.17	44.5	1.00	3.83
MCPB	1	3d	+	+	11	32.09	28.82	2.82	5.91
			+	-	5	52.2	32.2	4.00	4.60
			-	+	15	24.8	31.33	2.53	2.80
			-	-	6	6.33	31.67	1.67	3.67
MCPB	1	3wk	+	+	6	87.5	71.17	4.00	12.67
			+	-	6	77	87.5	3.33	5.83
			-	+	6	13.5	54.17	1.67	2.17
			-	-	6	9.83	50.83	1.67	4.17
Bentazone	10	3d	+	+	11	57.64	75.73	4.36	10.18
			+	-	6	63.17	94.83	4.67	13.00
			-	+	9	24.11	59.67	3.11	3.89
			-	-	6	11	50	2.33	4.67
Bentazone	10	3wk	+	+	6	86.5	72.83	4.33	13.33
			+	-	6	67	91.17	4.17	9.00
			-	+	6	24.67	52.5	2.83	3.50
			-	-	6	8.5	34.67	1.67	2.67
Bentazone	1	3d	+	+	12	62.17	68.83	4.50	10.33
			+	-	6	73.83	82.83	4.50	14.33
			-	+	8	38.5	57	3.13	7.88
			-	-	6	10.5	54.67	2.17	4.50
Bentazone	1	3wk	+	+	6	77.83	74.17	4.00	11.50
			+	-	6	75.5	68.83	3.83	11.00
			-	+	6	13.33	44.83	2.00	3.50
			-	-	6	8.83	36.33	1.17	3.67

Herbicide	Conc.	Time.	N	R	Reps	Average Shoot ht.(mm).	Average Root lgth. (mm).	Average Leafno. per plant.	Average no.of Lateral Roots.
Fusilade	10	3d	+	+	14	53.64	102.86	3.86	6.57
			+	-	5	52.6	77	4.00	9.00
			-	+	14	25.36	63.71	2.64	4.14
			-	-	6	9.83	56.5	2.17	4.33
Fusilade	10	3wk	+	+	6	60.83	74.83	4.50	7.50
			+	-	6	66.33	61.5	3.83	10.00
			-	+	6	19.5	50.67	2.50	3.50
			-	-	6	8.5	45.83	1.67	4.00
Fusilade	1	3d	+	+	13	75.54	89.8	4.46	11.54
			+	-	5	56.2	83.6	4.80	10.00
			-	+	13	21	63.46	2.23	3.46
			-	-	5	12.6	76.6	2.40	5.40
Fusilade	1	3wk	+	+	6	63.33	68.5	4.67	8.67
			+	-	6	61.33	60.83	4.33	9.50
			-	+	6	24.83	58	2.83	4.33
			-	-	6	9.83	45.5	1.83	4.83
Kerb	10	3d	+	+	12	3.75	22	0.25	0.00
			+	-	6	2.33	20.17	0.17	0.00
			-	+	12	3.58	22.17	0.50	0.00
			-	-	6	2.17	21.17	0.17	0.00
Kerb	10	3wk	+	+	6	74.5	73.83	4.50	11.50
			+	-	6	74.33	86.5	4.50	7.83
			-	+	6	42.17	59.33	3.50	7.83
			-	-	6	11.17	51.5	1.83	3.83
Kerb	1	3d	+	+	9	20.33	40.11	2.11	1.67
			+	-	6	56.33	66.83	4.00	3.00
			-	+	12	5.58	25.92	0.83	1.08
			-	-	6	5.33	29.33	1.33	1.17
Kerb	1	3wk	+	+	6	81.67	81.33	5.33	12.83
			+	-	6	73.17	75.67	4.33	10.67
			-	+	6	29.67	61.17	2.83	6.17
			-	-	6	12.5	47.5	1.83	2.67
Controls		3d	+	+	34	70.59	76.24	4.35	9.00
			+	-	22	73.32	72.64	4.36	9.23
			-	+	47	23.62	54.26	2.51	3.23
			-	-	21	9.57	47.52	2.14	4.00
		3wk	+	+	30	77.57	79.67	4.43	14.23
			+	-	30	72.83	71.83	4.17	12.10
			-	+	30	22.83	50.57	2.10	4.03
			-	-	30	9.33	38.47	1.40	3.40

Herbicide	Conc.	Time	N	R	Av. Shoot Fwt.	Average Fresh Wt.(g).	Average Dry wt. (g).	Av. Nodule Number.	Ethylene (micro-mols/ml /hour).
Paraquat	10	3d	+	+		0.00342	0.00033	0	0.000209
			+	-		0.00035	0.0002	0	
			-	+		0.0032	0.00026	0	0.00035
			-	-		0.0035	0.0002	0	
Paraquat	10	3wk	+	+	0.05	0.0048	0.00067	0	0.00057
			+	-	0.05	0.0042	0.0005	0	
			-	+	0.01	0.0069	0.00081	0	0.00027
			-	-	0.01	0.0055	0.0005	0	
Paraquat	1	3d	+	+		0.0048	0.00067	0	0.00057
			+	-		0.0042	0.0005	0	
			-	+		0.0069	0.00081	0	0.00027
			-	-		0.0055	0.0005	0	
Paraquat	1	3wk	+	+	0.08	0.14	0.0104	0.67	0.003699
			+	-	0.09	0.15	0.0116	0	
			-	+	0.03	0.05	0.0034	1	0.0244
			-	-	0.01	0.01	0.00162	0	
MCPB	10	3d	+	+		0.05	0.00434	0.08	0.000103
			+	-		0.06	0.005	0	
			-	+		0.02	0.0017	0.73	0.00158
			-	-		0.02	0.0012	0	
MCPB	10	3wk	+	+	0.09	0.14	0.0109	0.67	0.00045
			+	-	0.07	0.12	0.01018	0	
			-	+	0.01	0.02	0.01213	1.67	0.0046
			-	-	0.01	0.02	0.0023	0	
MCPB	1	3d	+	+		0.06	0.00535	0.45	0.000116
			+	-		0.1	0.0056	0	
			+	-		0.04	0.0031	1.73	0.00622
			-	-		0.02	0.002	0	
MCPB	1	3wk	+	+	0.12	0.19	0.01165	1.5	0.00202
			+	-	0.09	0.15	0.0113	0	
			-	+	0.01	0.02	0.00253	1.33	0.00251
			-	-	0.01	0.02	0.00252	0	
Bentazone	10	3d	+	+		0.13	0.00997	0.64	0.0056
			+	-		0.11	0.0106	0	
			-	+		0.03	0.0033	4.22	0.0169
			-	-		0.02	0.0027	0	
Bentazone	10	3wk	+	+	0.13	0.22	0.01487	1.5	0.00102
			+	-	0.09	0.15	0.0121	0	
			-	+	0.01	0.04	0.0052	2.83	0.01762
			-	-	0.01	0.02	0.0026	0	
Bentazone	1	3d	+	+		0.12	0.00944	1.83	0.00597
			+	-		0.13	0.0125	0	
			-	+		0.06	0.00544	3	0.01762
			-	-		0.02	0.003	0	
Bentazone	1	3wk	+	+	0.12	0.2	0.0145	2.33	0.0023
			+	-	0.09	0.14	0.0103	0	
			-	+	0.02	0.02	0.0028	1.83	0.0059
			-	-	0.01	0.02	0.00265	0	

Herbicide	Conc	Time	N	R	Average Shoot Fwt.(g).	Average Fresh Wt.(g).	Average Dry wt. (g).	Average Nodule Number	Ethylene (micro-mols/ml /hour).
Fusilade	10	3d	+	+		0.08	0.00722	1	0.00144
			+	-		0.09	0.0074	0	
			-	+		0.03	0.00298	2.14	0.00434
			-	-		0.02	0.0023	0	
Fusilade	10	3wk	+	+	0.06	0.12	0.01098	0.83	0.0027
			+	-	0.07	0.12	0.0121	0	
			-	+	0.01	0.02	0.0028	5.67	0.00729
			-	-	0.01	0.01	0.0023	0	
Fusilade	1	3d	+	+		0.16	0.0124	1.77	0.002085
			+	-		0.11	0.0086	0	
			-	+		0.03	0.00302	2.92	0.0047
			-	-		0.03	0.003	0	
Fusilade	1	3wk	+	+	0.08	0.12	0.0111	1.17	0.0015
			+	-	0.08	0.12	0.012	0	
			-	+	0.02	0.04	0.0038	2.67	0.0096
			-	-	0.01	0.02	0.00218	0	
Kerb	10	3d	+	+		0.01	0.00021	0	0.00008
			+	-		0.01	0.00082	0	
			-	+		0.01	0.00086	0	0.00017
			-	-		0.01	0.001	0	
Kerb	10	3wk	+	+	0.1	0.14	0.0123	1.83	0.000868
			+	-	0.09	0.15	0.0126	0	
			-	+	0.04	0.07	0.00665	3.67	0.01963
			-	-	0.01	0.02	0.002217	0	
Kerb	1	3d	+	+		0.03	0.0037	0	0.000039
			-	-		0.09	0.0075	0	
			-	+		0.012	0.0026	0	0.000083
			-	-		0.01	0.002	0	
Kerb	1	3wk	+	+	0.11	0.16	0.0134	0.67	0.00076
			+	-	0.1	0.16	0.01185	0	
			-	+	0.03	0.04	0.00415	2	0.00713
			-	-	0.01	0.02	0.00273	0	
Controls		3d	+	+		0.14	0.01022	0.97	0.002586
			+	-		0.13	0.0089	0	
			-	+		0.03	0.00356	2.66	0.01044
			-	-		0.02	0.002724	0	
		3wk	+	+	0.12	0.18	0.01497	1.4	0.005212
			+	-	0.1	0.16	0.01347	0	
			-	+	0.02	0.03	0.00365	2	0.010736
			-	-	0.01	0.02	0.002367	0	

Appendix 4. Data from pot study of herbicide toxicity toward *Trifolium repens/Rhizobium tri* symbiosis.

Herbicide	Soil water level. (WHC) (%)	Conc-entrat-ion.(x recomm. conc.)	Av. no. of nodules per plant plant.	Av.shoot fresh wt. per plant (g).	Av. root fresh wt. per plant. (g).	Av. dry wt. per plant. (mg).	Av. number of plants per pot.
Paraquat	100	0	6.905	0.215	0.02	15.39166	27
		1	4.62	0.15	0.02	0	0
		10	0.00	0.00	0.00	0.00	0.00
Paraquat	50	0	4.065	0.09	0.01	11.5835	27
		1	2.96	0.06	0.01	0.00	0.00
		10	0.00	0.00	0.00	0.00	0.00
MCPB	100	0	6.50	0.22	0.02	15.73526	26.6
		1	4.62	0.15	0.02	15.027758	26.25
		10	4.65	0.08	0.02	9.492775	22
MCPB	50	0	3.845	0.09	0.01	11.34644	25
		1	2.96	0.06	0.01	8.90565	22.
		10	2.85	0.06	0.01	8.90565	22
Bentazone	100	0	7.07	0.21	0.02	17.36296	26.6
		1	4.62	0.15	0.02	16.67455	27.25
		10	4.00	0.12	0.03	8.00645	15.75
Bentazone	50	0	4.14	0.09	0.01	11.1827	23
		1	2.96	0.06	0.01	5.1535	21.5
		10	2.38	0.02	0.00	4.5167	16.6
Fusilade	100	0	6.84	0.20	0.02	12.76856	26.6
		1	4.62	0.15	0.02	15.01418	26.75
		10	5.01	0.16	0.01	12.501925	26.5
Fusilade	50	0	4.12	0.095	0.015	12.3807	26
		1	2.96	0.06	0.01	11.634675	22
		10	3.15	0.07	0.01	9.790075	23
Kerb	100	0	6.64	0.21	0.02	14.012175	27
		1	4.62	0.15	0.02	13.9621	27.4
		10	6.31	0.19	0.01	13.154175	27
Kerb	50	0	4.34	0.095	0.015	12.9241	25.25
		1	2.96	0.06	0.01	9.9553	24.75
		10	3.88	0.09	0.01	12.62965	24.5

Appendix 5. Procedure for Acetylene Reduction.

1.Acetylene reduction method for *in vitro* experiments.

M^{c}Cartney bottles were used for incubating individual plants from *in vitro* experiments. Single plants were placed in each bottle which was then filled with N_2 and sealed.

Each M^{c}Cartney bottle incubation chamber was brought to a 4 % mixture of acetylene (C_2H_2) in N_2 by syringing out 4 % of the inert gas and replacing it with C_2H_2. When C_2H_2 was injected, the syringe was flushed several times to mix the gas inside the bottle. M^{c}Cartney bottles containing plants were incubated for 40 minutes in a growth chamber as described in section 1.2.4.

The atmosphere in each incubation chamber was sampled using a 1ml disposable syringe. M^{c}Cartney bottles were shaken and syringes flushed several times to ensure the sample taken was representative. 0.5ml samples were taken. Joints between the needle and the body of the syringe were sealed with Parafilm to ensure no leakage of the sample during storage. Syringe tips were stored under water until determinations could be made. 0.2 ml samples were injected into the Gas Chromatograph (GC). Controls and standards used were;

i.Two replicates of a standard mix of 10% C_2H_2 and 0.01% ethylene (C_2H_4) for callibration of C_2H_4 production values.

ii.A 4% C_2H_2 mix with N_2 identical to that present in the incubation chambers the level of impurity of C_2H_4 in the C_2H_2 used.

iii.A nodulated plant incubated in an atmosphere of N_2 only to indicate the presence and amount of exogenously produced C_2H_4.

A field operation GC was used for C_2H_4 determinations. The detector is a Taguchi Model TGS-812 gas sensor (Figaro Engineering Co.,Toyonaka city, Asaka 560, Japan) consisting of a small filament heater surrounded by a sintered semiconductor. When the filament is heated in air in the presence of a combustible gas, the decrease in the resistance of the semiconductor is measured with an ammeter. The carrier gas is compressed air maintained at a pressure of 35KPa or at a flow rate of 20 ml/minute. A conventional column for use with standard gas chromatography was used, consisting of a 1cm X 2 metre stainless steel column packed with Porapak N (80-100 mesh). This system gives a retention time for C_2H_4 of 55 seconds and for C_2H_2 of 137 seconds.

2.Acetylene reduction method for pot experiments.

Plants were removed from pots and shaken free of soil. The total number of plants from each pot were placed in Agee preserving jars with a volume of between 570 and 590 ml. The volume of each jar was taken into consideration when calculating gas mixtures.

Agee vacuum seals with holes of approximately 1cm diameter drilled in

inside the jars was brought to 4 % C_2H_2 by removing 4 % of the atmosphere inside the sealed jar and replacing it with the same amount of C_2H_2. It was decided not to replace the atmosphere inside the jars with an inert gas as this has been shown to be unnecessary (Mague and Burris 1972).

Jars were incubated for 1 hour in a growth room at 22°C. Jars were shaken to ensure homogeneity of the atmosphere and 0.5 ml samples were taken. Samples were flushed through the syringes 3 times in order to take a representative sample. Joints of syringes had been sealed with Parafilm and syringe tips were placed under water to prevent leakage prior to injection into the GC. Two 0.2 ml samples were injected into the GC from each syringe. Standards and controls used were as detailed in Appendix 5, Section 1.